

**nature***July 22, 1976*

## Out of work in Argentina

THE letter (page 253) from more than 60 Italian scientists drawing the scientific community's attention to the situation in Argentina is a grim reminder of the difficulties surrounding university and government institutional work on a continent which for many of us in the Anglo-Saxon world is still remote and in some way unreal.

The intellectual in Argentina has never been as secure as his counterpart in West Europe and North America. Universities have been watched very closely by successive governments, and the appointments of rectors and deans have often been purely political affairs. As a result there has been a steady trickle of emigrants to more secure territory—a trickle that has sometimes swelled to a flood (in 1966, for instance, 1,100 found themselves out of a job). The turnover in university staff has been accordingly immense; one academic in Britain, who has fairly strong ties with Argentina, remarked that almost every Argentinian scientist he had known had been forced, at some time, to leave. The quality of young people coming up through the major universities is, it is widely agreed, suffering as a result.

Argentina's problems in recent years have, of course, gone much deeper than instability in universities and there was genuine hope when General Videla came to power that he would be able to act against the terrorism which had led to so much indiscriminate killing. To a certain extent he has succeeded because terrorism by the left has been sat on very firmly; however, right-wing activities do not seem to have been significantly restrained. The bewildering variety of competing security organisations and the divisions within the armed forces add to the complexity of the situation. An exiled Argentinian commented that knowing how one stood in La Plata would not necessarily make one safe in Buenos Aires, and so on. It is small wonder that universities have become places of fear and that gun-carrying soldiers are in evidence.

If recent dismissals are, in general, politically directed against the left, they are not universally so; several well known right wingers have also been relieved of their posts. What seems to have happened is that the government, faced with a truly depressing economic situation requiring urgent international support, and forced to make major cuts in public expenditure, has used the opportunity to thin its ranks of those who have taken up certain political stances in the past. Those who have steered clear of politics or who openly support the Junta can survive, though in somewhat straitened circumstances.

It used to be that, with families widely spread over South America, people in trouble could move over to Chile, Uruguay or Paraguay. Indeed Uruguay was often called the Switzerland of South America. No longer; the Uruguayans in their zeal to stamp out the Tupamaro guerillas seem to have 'processed' an extraordinary large proportion of the population and to have allowed torture to become rampant. The fate of the mathematician Jose Massera who has been imprisoned since last October and has suffered some grievous injuries is likely to deter most academics from choosing to move over to Montevideo.

The pressure, then, is really on the wider community of scientists to continue to absorb where necessary or, even more, just to keep in touch—a plea made most simply and expressively by the writers of the letter. From the Europe–North American axis it is very easy conveniently to forget the difficulties of doing science elsewhere, to look down on scientists struggling with poor facilities and heavy teaching loads, and to ignore colleagues off the axis when passing round the gossip, the early warnings, the bits of advice. Even well endowed Australians, with regular jet flights to the Northern Hemisphere, know that. How much more so does the problem afflict Argentinians, and how much more must we simply not stand by uninterested. □



## Why are we waiting?

ALTHOUGH Mr Mulley, UK Secretary for Education and Science, has had the report of the Williams Working Party on Genetic Manipulation of Micro-organisms on his desk for well over a month, it is due to remain there for at least a "few more weeks". Until then scientists will be in the dark over the details of the code of practice that will apply to recombinant DNA research in Britain. There are several possible excuses for Mr Mulley's tardiness. None of them is good enough.

The Williams Working Party had its origins in a statement published by Professor Paul Berg and his colleagues in July 1974 in which they expressed concern at some of the possible consequences of the research they themselves had pioneered. The improbable pathway to disaster that they foresaw involved the escape from laboratory containment of bacteria whose genes had been experimentally recombined with genetic material from another organism, the survival of those bacteria in the outside world, their colonisation of the human intestine and the expression of their foreign genetic component to the detriment of the human host.

That scenario was taken seriously enough on both sides of the Atlantic that most scientists have since held to a voluntary moratorium on suspect research. American concern, latterly under the auspices of the National Institutes of Health, culminated four weeks ago in the issue of a complex series of guidelines. The British wheels were set in motion in July 1974 with the Ashby Working Party, convened by the Advisory Board for the Research Councils, which reported in December of that year. Seven months later Mr Mulley set up the Williams Working

Party to produce a code of practice and to consider the establishment of a central advisory service for laboratories carrying out the procedures in question.

The Williams Working Party report is unlikely to contain many surprises. It will not recommend the proscription of any particular experiments but will suggest the precautionary measures that are appropriate for various categories of research. Few specific experiments will be quoted and no attempt to quantify their dangers will be made. The report is expected to endorse Ashby's suggestion of a central advisory service and to recommend how it might operate.

The scientific community anxiously awaits the report and Mr Mulley's reactions to it. The longer they have to wait for a go-ahead on experiments which in some cases have been on ice for two years, the worse (whatever the precautions suggested) will become the atmosphere of frustration and suspicion that has gradually built up. Inevitably moratorium-breaking has already occurred, and the consequences for those involved have on occasion been unpleasant.

Why then is Mr Mulley keeping us waiting? One possibility is that he is reserving the right to modify his reactions in the light of the response at home and abroad to the American guidelines. If so the delay could well be lengthy in view of the recent clash between Cambridge (Massachusetts) City Council and Harvard University. The trouble arose when the Mayor of Cambridge attempted to block plans to build a high containment laboratory within the Harvard Biological Laboratories; at the moment there is a three-month moratorium during which the

council will review the position before deciding on the future of Harvard's genetic engineers. Similar problems are expected in other American cities. Although there is a reasonable chance that such clashes will be avoided in Britain, the longer Mr Mulley delays, the more likely they are to occur.

A second reason for delay may simply be the need for extensive briefing and consultation between departments. If so Mr Mulley must lavishly oil the cogs of bureaucracy. Most likely the delay is due to his consideration of the introduction of statutory controls of recombinant DNA research. Although the Working Party is thought to have been reluctant to recommend statutory controls, they may have made some suggestions in that direction because of forceful representation from the unions. The same pressures, which clearly must be respected since the unions represent those most likely to be directly carrying out the research, may now be holding Mr Mulley back.

The British code of practice, with or without statutory backing, could be very influential. In contrast to the American code, which applies only to NIH-supported research, the British code will apply to all academic institutes and probably also to industry via the Health and Safety Executive. If that breadth is matched by an authoritative depth and practical recommendations, the code could well be adopted by other European countries. That could happen through the European Molecular Biology Organisation which meets to consider the matter on August 12. It would be a great pity if the British code had not emerged by then. □

## Solar energy breeders

*Malcolm Slessor and Ian Hounam of the Energy Studies Unit at the University of Strathclyde, Glasgow, offer an opinion on a largely ignored aspect of solar energy*

SOLAR energy has not been taken seriously as a solution to our energy supply problem and for good reason. It requires considerable land area and the sun has an unhappy knack of ducking behind clouds or withdrawing into a winter solstice. But there is one aspect of solar energy that is significantly in favour of its large scale development, an aspect which has so far received scant attention. This is the potential for solar energy devices to breed energy. No other device can do that. Yet, given the right technology, we calculate that with an initial invest-

ment by 1980 in 1 MW of solar power, it would be possible for solar energy to provide 90% of the world's energy needs within 40 years, without placing any burden upon existing energy resources.

The importance of this claim can hardly be exaggerated. If a solar breeder system was in operation, nearly all the energy from North Sea oil could be devoted to current needs and not, for example, to building alternative energy systems that will be needed when oil runs out. If we have a viable solar breeder system, world-

wide, then once that system has grown large enough to satisfy the bulk of world demand the price of energy will fall to a trivial value, because after all the sun is a free good. Such a fact would utterly change the basis of a manufacturing or even a service orientated economy.

What, then, is an energy breeder? It is a device which "breeds" capacity to generate useful energy without consuming energy stocks. In this sense nuclear "breeders" are not breeders but extenders, increasing the potential energy of uranium by roughly 100/0.7, where the 0.7 is the proportion of Uranium 235 occurring in natural uranium. But when a solar capture device (SCD) (or any solar induced device such as a wind or wave gener-



ator) delivers in its lifetime more energy than needed to maintain and rebuild itself, then some of that surplus can build more SCDs, thus breeding energy.

Breeding success depends on the pay-back time of the initial energy of manufacture, and energy analysis provides us with a means of assessing it. Through a method known as dynamic net energy analysis, in which energy required to construct and service an energy production or transformation system is set against the system output as a function of time, one can test various breeding scenarios. Unfortunately the energy investment data so far produced on constructing solar energy devices (other than solar heat devices) is unconvincing, and tends to consider only optimal sunlight conditions. Moreover, all too often figures include only direct energy costs, and the more obvious ones like the energy requirements of structural steel and glass. No comprehensive results have been published enabling one to estimate the gross energy requirement to build and operate an SCD in an average situation.

Our own tentative energy analysis of 1972 photo-voltaic technology has shown it would take about 40 years output to pay for the energy investment, and since its expected life may be about 30 years it is clearly a sink, not a breeder. However, the new technology of ribbon melting of silicon looked like reducing that to 10 years. In recent discussions with the Office of Technology Assessment in the USA we have learnt of systems which are said to require only a two-year pay-back time. This clearly provides tremendous breeding potential.

If a system could grow while also satisfying its own energy needs—that is to say the energy for construction and operation—it then places no burden on the existing fossil and nuclear energy infrastructure, and the only charge upon the building programme is the labour and management and the land needed for expansion of the system.

Choosing a datum of 1980 for the initiation of the programme of breeding, with an initial 1 MW output capacity SCD system, we examined a range of pay-back times varying from the pessimistic result at Strathclyde (10 years) to the optimistic views of the Office of Technology Assessment (2 years) for various investment policies, to show that energy breeding is a possibility even with as large a value of pay-back time as 10 years, though the rate of breeding is too low to be of real interest. Thus for a policy of 5% investment in new capacity, by 2030 the programme would only have developed an output capacity of

12.2 MW (as against the original 1 MW in 1980) and a net output power would be a mere 4.3 MW—reflecting a system doubling time of about 14.5 years. Yet even that is greater than the global rate of growth in energy demand.

On the other hand, if we accept the optimistic value of pay-back time of 2 years being claimed in some quarters, an SCD breeding programme could be spectacularly successful. For example, with an investment fraction of 40%, an initial output capacity of 1 MW in 1980 would have grown to an output capacity of  $8.8 \times 10^6$  MW by 2020, giving a net power output to the non-solar energy sector of the economy of  $1.7 \times 10^6$  MW. By this time the doubling time of the SCD system is only 3.2 years, a rate of growth of energy supply higher than the most horrific energy demand scenarios.

Of course by no means all problems are solved. Though the programme provides its own energy requirements, there are still the problems of land availability and manpower. Some will point out that there is also a need for steel, glass and so on. But of course, the energy analysis has taken that into account through the energy required for construction. Nevertheless, there remains the task of assessing whether the necessary industrial infrastructure to sustain the solar programme can be created, a question we do not attempt to answer here. We merely set out to show that, given the appropriate technology (low pay-back time), a solar capture system could breed itself, and hence in time solve the global energy supply problem. The devices with larger than two-year pay-back times are less satisfactory. Clearly, then, in view of the tremendous opportunities presented by a device with a two-year pay-back time, a massive research programme is justified.

The SCD system output capacity could match even the most ambitious forecasts of demand ( $4.5 \times 10^6$  MW) within 40 years of initiation. From an initial recycle fraction of 80% to new investment, the construction programme falls away to 7% in the 40th year. In net energy terms this is a lower figure than found in our present fossil and nuclear fuel industries. It would be consistent with a price, in current terms, of around £100 per kW for photo-voltaic collectors.

It is interesting to consider what happens to the cost of conventional fossil and nuclear fuels if such a programme took place. By the year 40 (2020), solar energy could so nearly supply all energy needs that oil, gas, coal and nuclear sources will have lost much of the scarcity value they now enjoy. True, oil and gas will

remain valuable for their feedstock potential, but nuclear reactors could be rendered redundant and coal mining unnecessary, save as a source of carbon. In such a situation presumably the price of energy falls to a trivial value.

This is offset by the land requirement of SCDs. Taking the 40 year level of output of  $4.2 \times 10^6$  MW, and assuming that the two-year pay-back time has been achieved, with collection of 20% efficiency, a huge land area is required, around 30,000 km<sup>2</sup> (equivalent to about 12% of the British Isles), but it is spread world wide. However, if the same period of two-year pay-back time applies to solar generated SCDs such as wave and wind power generation, then land requirement falls considerably.

In order to effect the programme, a cumulative energy investment of  $1.7 \times 10^6$  MW years of energy would have been used—that is about  $25 \times 10^9$  tons coal equivalent. This is a huge quantity of energy, and reflects a huge amount of material and man power. None of that energy, however, would have come from global energy resources, but from within the programme. Secondly, the basic problem facing all industrialised societies is the need to build a new energy infrastructure within the next 30–50 years. When we examined the task of doing this for the UK imagining an eventual replacement of fossil energy by nuclear and some solar, we found that the massive energy investment called for placed restrictions of UK export of North Sea oil, and a serious limitation on UK economic growth, for the energy investment had to come from existing energy resources and transformation devices. The SCD breeding system, however, permits a parallel economic development without this strain being placed on existing energy resources.

In reality an SCD breeding programme would differ considerably from the simple model presented here. For one thing much of this growth would be dispersed—some citizens for example, building wind generators in their back yards. Technology would change rapidly, and pay-back times would vary according to the sophistication and purpose and location of the device. Much solar energy could be turned directly to heat, through collectors. It is still too early to find whether the torrid dunes of the Sahara will pay a more or less important role than the ceaseless waves of the North Atlantic and South Pacific. But given a short pay-back time and an integrated breeding and development programme, prospects for a low cost energy, nuclear-free world do look good. □



# Modelling the world, again

David Spurgeon in Ottawa looks at two recently published studies of the world's future

WHEN it was published in 1972, *The Limits to Growth* was a kind of landmark, crystallising in a mathematical model the views of those who believed that the world had come to a turning-point in its profligate use of resources, its growing pollution of the environment, and its inexorable population growth. Its message was basically a simple one: that if current trends continued, disaster was certain, because resources which were finite would soon be exhausted, pollution which was increasing would soon become intolerable, and the press of population which was exponential would soon outrun living space.

For many the book was authoritative because it was backed by computer studies—and perhaps because it reinforced existing fears or prejudices. But given its assumptions, its conclusions seemed rather obvious, and it provoked a rash of criticisms, as well as a number of similar studies. Whatever else may be said of it, though, it succeeded in raising the public's consciousness of some of mankind's major problems and prospects.

Two recently published studies\* deal directly with the kinds of problem examined by *The Limits to Growth*: one, *Catastrophe or New Society?*, arose as a specific response to it, while the other, *The Next 200 Years*, could be said to have been undertaken in response to its underlying philosophy. Both disagree totally with the assumptions and conclusions of *The Limits to Growth*, while at the same time proceed from assumptions totally at odds with each other. What they have in common is a basic conclusion that the limits to growth—if any—are not physical, as suggested in *The Limits to Growth*, but sociopolitical, and that mankind is not doomed either to a no-growth economy or to disaster but is capable of providing a worthwhile and satisfying future for all. Where they differ is in how such a future could be brought about.

\**Catastrophe or New Society? A Latin American World Model*, Amílcar O. Herrera, Hugo D. Scolnik, Graciela Chichilnisky, Gilberto C. Gallopin, Jorge E. Hardoy, Diana Mosovich, Enrique Otieza, Gilda L. de Romero Brest, Carlos E. Suárez, and Luis Talavera (IDRC-064e; International Development Research Centre, Ottawa, Canada; 1976). *The Next 200 Years: A Scenario for America and the World*, Herman Kahn, William Brown, and Leon Martel, with the assistance of the Hudson Institute (William Morrow and Company, Inc., New York; 1976).

The basic premise of *The Next 200 Years* is that what we need is not less growth, but more—and along pretty much the same lines as at present—at least until world-wide economic development has been completed. After that growth rates will slow and many economies will reach a more or less steady state. *Catastrophe or New Society?*, on the other hand, sees achievement of a good life for all, particularly those in developing countries, as impossible without certain fundamental changes in society. What is wrong with the arguments of Meadows and his ilk, in its view, is that they accept, "in a totally uncritical manner, the central values of society as it now is." Herrera and his colleagues maintain that the major problems facing society

are based on the uneven distribution of power, both between nations and within nations. The result is oppression and alienation, largely founded on exploitation. The deterioration of the physical environment is not an inevitable consequence of human progress, but the result of social organisations based largely on destructive values.

In its place must be constructed a new type of society—socialist, egalitarian, fully participatory, and non-consumerist.

Both books project the future from a very clear ideological base, though the base is made more explicit and acknowledged more directly by Herrera's group than by Kahn's. Both are essentially utopian (Herrera's admittedly so, Kahn's not) and suffer from the basic fault of all such modelling of enormously complex systems: oversimplification, and the necessary exclusion of all sorts of potentially important variables that either cannot be accurately quantified or cannot be foreseen.

Kahn and colleagues argue that "the current malaise", as they call it, is propelled by a combination of compassion and guilt for the plight of the world's poor and the coincidental occurrence of world-wide crises in the supply of food and energy. They believe that the movement toward this view "has gone too far", and argue that there is both need and opportunity for economic growth—that precisely because America and the rest of the developed world use resources so intensely, there will be stimulation, not depression, of the economies of developing nations. Using what seems, as far as they reveal it, to be questionable data, the Kahn group suggests current population

growth fears may be nothing much more than "an amusing episode in human history". Looking backward from 2176, as they pretend to be able to do, they say "the temporary nature of the current population phenomenon becomes very obvious".

Similarly, energy supplies are no problem. Examining current fossil fuel reserves (on the basis of a 1973 US Geological Survey that has since been withdrawn by that agency and drastically revised downwards) and the prospects for other energy sources, both conventional and non-conventional, developed or not, the authors conclude:

Except for temporary fluctuations caused by bad luck or poor management, the world need not worry about energy shortages or costs in the future. And energy abundance is probably the world's best insurance that the entire human population (even 15–20 billion) can be well cared for, at least physically, during many centuries to come.

The same goes for raw materials: "... there is an abundance of raw materials for future generations as well as the present one, and ... the more man develops economically and technologically, the more there will be for all of humanity."

And as for food, "the position we argue is that, except for the occasional regional fluctuations caused by natural disaster, inappropriate policies or the misapplication of resources, the long-term prospect is for adequate food supplies". Within 200 years, the authors say, it will be possible to increase world food consumption to the level of the United States today. What will happen if "inappropriate policies or the misapplication of resources" continues to be the rule, as it is today, is not dealt with. Yet that, as the authors of the Latin American world model recognise, is precisely where the real problem lies.

To give them their due, the Kahn group do recognise this. They go so far as to state that

any limits to growth are more likely to arise from psychological, cultural or social limits to demand, or from incompetency, bad luck and/or monopolistic practices interfering with supply, rather than from fundamental physical limits on available resources.

What seems odd is their faith in things as they are, and their acceptance that they will continue that way.

... Large income gaps between nations could persist for centuries ... there are few peasants, workers or even businessmen in developing nations who care much about gaps (whether arithmetic or geometric), no matter how much intellectuals, academics and some businessmen may profess to. The major objective of most people is to increase their own safety and improve their own standard of living and their own capabilities. When they make comparisons, it is

usually with others at their socioeconomic level or with those who have recently been at their own or a lower level.

By such a yardstick, much of the Third World should have nothing to worry about. The Kahn group sees communist Asians with a GNP per capita of about \$1,000 by the year 2000, and about half of the Third World with about double that. A desperately poor group, mostly in India but also in parts of Latin America and Africa, could—even with “reasonable” policies—attain only an average of about \$200 per capita by the year 2000. But in another hundred years almost all societies should have a GNP per capita of more than \$2,000. The fact that others may have 10 to 20 times more is, in their view, of no consequence.

The Latin American world model is based on the premise that, in the new society proposed, the production system has the satisfaction of basic human needs as a main objective. These needs are nutrition, housing, education and health. The mathematical model was constructed around the satisfaction of these needs, differentiated into five sectors: nutrition, education, housing, capital goods, and finally, consumer goods and other services. It was built to test the physical viability of the proposed society. This involved determining the period of time needed and the conditions under which the different regions could satisfy the basic needs to given levels.

The level of food was taken as 3,000 calories and 100 grams of protein per day (3,200 calories in advanced countries); of education, 12 years of basic education between the ages of 7 and 18; and of housing, one house per family. In the developed countries, the minimum dwelling would have an area of 70 square metres for an average family of 3.5 people, costing \$4,900 to build. For Africa and Asia, the standard house was more modest because of the current desperate housing situation: 7 square metres per person, at \$23.40 per square metre in Africa and \$11.20 in Asia. Housing conditions would reach developing country standards in 20 years.

The annual cost of education per student in underdeveloped countries reached a maximum of \$150, and an aggregated yield of 4 tonnes per hectare per year was assumed in agriculture. In developed regions, economic growth would be restricted to 1–2% when GNP per capita exceeded \$4,500 annually. And for underdeveloped regions, once basic needs were satisfied, growth in GNP per capita had to be at least 2%. This is to gradually reduce the gap between the developed and underdeveloped regions.

What did the model predict? For

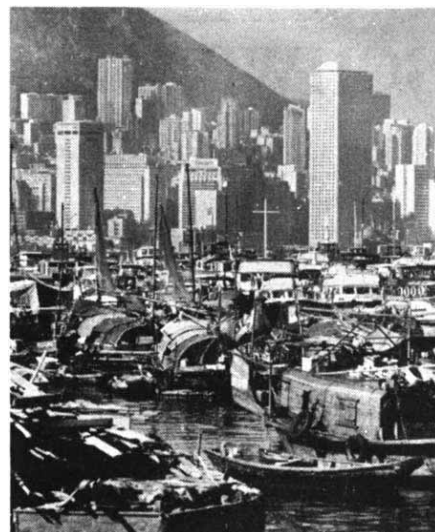
developed countries basic needs were shown to be satisfied in the first few years of the computer run (which ended in the year 2060). GNP per capita reached \$9,470 in that year (slightly less than Kahn's projection). Latin America could fulfill its basic needs in the early 1990s, and its GNP per capita would rise from \$372 in 1960 to \$5,746 in 2060. Interestingly, the population growth rate, which was 2.8% in 1960, decreased as general well-being improved, and was only 0.43% by 2060, approaching stabilisation. The consequence for Latin America, then, is that it could satisfy its whole population's basic needs within a generation of the implementation of the proposed policies.

It would take Africa longer: that continent could satisfy its basic needs by 2008. GNP per capita, \$137 in 1960, would reach only \$559 in 2008, and \$2,657 in the last year of the run. Thus if the policies proposed were applied, Africa could satisfy the basic needs of its population with 30 years after 1980, according to the model.

Asia's position is very different. According to the model, basic needs could not be satisfied by 2060. Food consumption of 2800 calories could be achieved by 1992; that level would be maintained until the mid-2020s, but would decline after that until levels reached were incompatible with survival. Nor were housing needs achieved, with only 0.82 house for every family in 2040. Education would be the only basic need totally satisfied by 2040.

“The problem in Asia arises in the food sector,” says the report. “By 2010, all available land is being cultivated. Thereafter, economic effort in the sector is devoted to increasing livestock and fisheries. This, however, is not enough to feed the growing population adequately”. If agricultural yields were increased from 4 to 6 tonnes per hectare, however, basic needs could be satisfied. GNP per inhabitant—\$89.70 in 1960—reaches \$1,516 in 2060. But food remains a problem, for in the mid-2030s the remaining land has been used, and “it is inevitable that some years after 2060 Asia will not be able to feed its inhabitants adequately.”

Finally, a run was carried out to test the effect of cessation of technological progress in the evolution of the regions involved. It was supposed that there would be no more technological progress after the year 2000. This produced significant effects. In Latin America, it increased the time necessary to satisfy basic needs, increased the population growth rate, and reduced GNP per capita (from \$5,746 in 2060 to \$1,173). In Africa, as in Asia, minimum objectives could not even be achieved: the economic system collapsed. Only



*Two views of the limits to growth?*

food reached the target level in Africa, and then only for a brief period. In Asia, none of the basic needs were met. Thus technological progress is seen as essential for liberation from underdevelopment and misery.

International aid is not seen by either the Kahn or the Herrera study to be a decisive factor in raising the level of well-being of Third World countries. Under conditions currently prevailing, even increased international aid would only contribute to increased spending by privileged sectors and have little or no effect on the living standards of the majority of the people. In direct contrast to the Kahn hypothesis, Herrera and his colleagues maintain that economic growth without concurrent drastic modification in income distribution would not achieve an adequate level of well-being for the entire populations of developing countries.

Many people tend to argue that studies like these are nothing more than a form of wish fulfillment; that they will increase the skepticism of laymen ignorant of the true nature of future studies and mathematical modelling because they seem to indicate that computer studies can be used to prove any thesis their authors wish; that their obvious political naiveté or ideological bias makes them trivial, if not dangerous. Others will see them as useful contributions to a continuing debate—a debate that must be kept going if solutions are to be found for the problems they deal with. Perhaps they will have done all they can if they succeed in showing, as both studies are intended to do, in the words of the Herrera group, “that the fate of man does not depend, in the last instance, on insurmountable physical barriers but on social and political factors that man must modify.” □



## BRITAIN

## SGHWR: green to amber?

*Allan Piper reports on uncertainties surrounding the next generation of British nuclear reactors.*

GROWING doubts about the future of the British-designed Steam Generating Heavy Water Reactor (SGHWR) have led the House of Commons Select Committee on Science and Technology to call an inquiry into progress on the reactor system, now at the end of the design stage. The committee two years ago successfully urged the controversial adoption of the SGHWR for the next phase of Britain's nuclear energy programme. It has now expressed concern over "the widely reported intention of the Secretary of State for Energy [Mr Anthony Wedgwood Benn] to review the SGHWR project in the near future".

In fact Mr Benn is currently awaiting reports on technical and economic aspects of the SGHWR from the United Kingdom Atomic Energy Authority (UKAEA), which has recommended a "take-stock" review, and from the Central Electricity Generating Board (CEGB). Parliamentary questions and statements from key leaders of management and unions within the nuclear industry have fed speculation that the reactor may be abandoned as part of the government's present review of public expenditure.

Mr Benn is expected to appear as an early witness in the select committee inquiry, probably before the forthcoming Parliamentary recess. The heads of the nuclear industries and the CEGB will be invited to appear at a later date.

Disagreement over the choice of the SGHWR as the commercial successor to the Advanced Gas Cooled Reactor (AGR) has continued since the initial 4000 MW ordering programme was announced by Mr Benn's predecessor in July 1974. The go-ahead came then

in spite of opposition within the nuclear industry and the CEGB's preference for a much larger programme using mostly American Pressurised Water Reactors (PWRs). The SGHWR was eventually favoured on safety grounds even though estimated construction costs were around 10% higher.

Officially, the SGHWR still has the green light. But there are amber tinges. The PWR has been given a clean bill of health by the Chief Scientist at the Department of Energy (DEN), while technical difficulties have pushed design costs of the SGHWR above original expectations. Following a major design review in the middle of 1975, and a further revision earlier this year, the CEGB has calculated that the SGHWR could eventually prove more costly than equivalent coal-fired capacity.

This week Mr Benn spoke in Parliament of the new activity on the American reactor front. Last month he admitted that the UKAEA had recommended a careful look at progress on the SGHWR project, and hinted that once reports have been received the reactor's future will be considered in the light of recent changes on the energy scene. The CEGB, which currently has 15,000 MW of new generating capacity either under construction or ready for commissioning, will probably indicate that with the present slump in electricity demand the first commercial SGHWR planned for Sizewell in Essex is no longer urgently required.

Ditching the project would mean the loss of political face and perhaps more, but it would also avoid economic and technological complications. Development of the SGHWR is not regarded as a crucial step along the path towards fast breeder reactor (FBR) technology, while the export potential of the reactor remains limited; most other

countries are expected to opt for the comparatively well proven PWR. Moreover, imported Canadian heavy water, needed to moderate the SGHWR, is likely to prove unexpectedly expensive.

Within the nuclear industry there is feeling that buoyancy can be maintained without the SGHWR if further commercial AGRs can be ordered. The first of the two AGR reactors at the 1,250 MW installation at Hinkley Point in Somerset is now running successfully up to full power, bringing confidence that the much-publicised developmental hitches can now be left behind. Additionally, there are hopes that with a go-ahead for the first commercial FBR this autumn, fast breeder technology could lag only three or four years behind SGHWR technology.

If the SGHWR is abandoned Britain could buy herself into PWR technology and establish an export base under licence. Japan and the USA, and several European countries, however, already have strong footholds in the market, which means that Britain would probably be restricted to the manufacture and export of component hardware. Mr Benn's junior, Alex Eadie, is meanwhile stressing that any reversal of SGHWR policy would not be at the expense of British technology.

Last week the DEN would only reaffirm that Mr Benn was awaiting reports from the UKAEA and CEGB. The UKAEA says simply that it is carrying out an appraisal which will be made available to Mr Benn in the near future. The CEGB is giving no outward sign that any imminent interruption to the SGHWR project is foreseen, and does not expect to have reliable cost figures ready for Mr Benn before next spring. An initial safety report is awaited from the Nuclear Power Company, responsible for design; this is not expected to arrive at the CEGB until next month, and is to be followed in turn by more detailed specifications in April 1977. □

## CANADA

## Towards a conserver society

*Like other industrialised countries, Canada must look to conservation as an important element in its future energy strategy. This point has received considerable emphasis from an important quarter, as David Spurgeon reports from Ottawa*

ENERGY conservation seems to be the major pre-occupation of the Science Council of Canada in its tenth anniversary year, judging by the statements

and publications emanating from its members. The most recent came from the chairman, Josef Kates, who is president of a Toronto firm of systems analysts. "Energy conservation is not an option—it is a necessity," he says in the council's annual report. "... The highest priority in any comprehensive national energy policy should be a wide-ranging, imaginative energy conservation effort. Such an effort must involve every individual and every organisation, private or public."

Dr Kates reinforced his points at a press conference given when the report was published. The reason for his concern is that Canada is the "second most energy intensive country in the world, and energy shortages could threaten the very fabric of our way of life." He says that if Canada's energy requirements also double by 1990, as is currently forecast, the needed capital investment could be as high as \$20 billion—or about 5 to 6% of GNP between then and now. This is some 50% higher than the 3.5% average between 1950 and 1975, and the interest rate on this capital is likely to be



from 50 to 100% higher than in the 1960s.

This may mean that by the 1990s energy costs will be twice or three times what they are today, and if per capita consumption is also 50 to 100% higher, energy costs could require from two to four times today's proportion of personal income. Canada's historical advantage in such energy-intensive industries as forest products, mining, agriculture, metallurgical and chemical industries could therefore be seriously attenuated. And if primary industries weaken, Canadians could not look to the secondary sector for compensation.

Dr Kates proposes that a much higher priority be given immediately to a comprehensive energy conservation programme, and that Canada must now begin to develop the energy technologies that will permit her to achieve a stable energy use pattern in the long run. This means exploiting renewable energy forms such as solar, wind, geothermal, biomass and tidal power. A major programme of research and development, of the kind first proposed by the Science Council in 1968, must be undertaken "to demonstrate the feasibility of alternative energy technologies."

A recent investigation indicates that "if we were able to hold Canadian energy consumption at present levels and continue to use existing or moderately-expanded hydro-electric capacity to provide approximately 25% of our energy needs, the remaining 75% (now met almost totally by non-renewable hydrocarbons, oil, gas and coal) could be provided within the next 50 years or so by renewable resources such as solar, biomass and wind energy."

At the press conference Dr Kates described the Conserver Society study the council's largest and most important project. The concepts behind this study, outlined in the "Statement of Concern" published in *Science Forum*, are summed up in the comment that "we face a transition from a 'consumer society' to a 'conserver society'"—that is, to a society which included among its features "doing more with less," "a greater appreciation of external or social costs," a return to the habits of thrift, saving and avoidance of waste for society as a whole, and so on.

"The transition to a Conserver Society," the Science Council paper says, "does not mean that we are going into a period of austerity and shortages. In fact, it is the reverse. Only by adopting a more rational and conserving approach to the common energy, resource, and environmental pool that sustains us all, can we ensure a continuing high standard of living for future generations." □

## THIRD WORLD

# Talking about technology transfer

*The transfer of technology from developed to underdeveloped countries, a subject examined at UNCTAD IV at the end of May, was also discussed at the recent World Employment Conference. Robin Sharp reports*

SCIENCE and technology, said the man on the rostrum, could increase production everywhere "on a scale that would make poverty unnecessary as a global phenomenon within the foreseeable future." The speaker was a senior official of the United Nations Development Programme, addressing last month's World Employment Conference in Geneva.

But for many people his otherwise heartening words raised questions. How is the scientific know-how and technology of the rich to be adapted and made relevant to the needs of the poor? How are the benefits of these high-cost, skill-intensive fields to be transferred to those possessing limited skills and no hard currency? And how, at the end of the day, can it be ensured that the transfer does not enrich 10 men at the expense of a thousand others? Among the many dilemmas of development these are some of the most intractable, and the debates in Geneva proved it once again.

Some of the figures can be frightening. In the industrialised countries, the average investment required to create one job is in the region of \$5,000. The developing countries, with most of the world's poverty, need to create five times as many jobs per million population in order to meet the basic needs of their people, with only one-tenth of the per capita income available to do it. Hence, on a rough rule-of-thumb calculation, an average investment of \$250 per job is what they can afford. There is little science and less technology to be had for that kind of money.

On this fundamental issue, delegates to the World Employment Conference found themselves deeply divided. At one end of the spectrum, speakers denounced the appropriate technology movement as a neo-colonialist strategy designed to perpetuate the supremacy of the rich countries. In more measured terms, the French Minister of Labour also questioned the international division of labour proposed to the conference by the International Labour Organisation (ILO), whereby

developing countries would specialise in the production of everyday consumer goods, using less sophisticated technology and unskilled labour, while the industrialised countries would concentrate their economies even more in the high-technology sectors.

Put more favourably, however, what the ILO was proposing in its report to the conference was a Basic Needs Strategy in which much greater effort in the field of appropriate technology was seen as essential for maximising the use of labour in the Third World. Given the present level of 300 million unemployed or underemployed in the developing countries, and their estimated need for 1,000 million new jobs over the next 25 years, the ILO strategy is to many the more persuasive formulation. With it has to be swallowed a slower rate of national growth, but in most developing countries the alternative—in terms of human and social cost—would be even less palatable.

Among Third World delegations, the Latin Americans in particular were convinced that they should "continue to incorporate the most advanced technology into those activities where it was necessary in order to eliminate the technological gap." At their preparatory meeting, by contrast, the African group had strongly endorsed the need for more work on appropriate technology as a matter of priority.

In the end, it was the western industrialised countries which blocked the ILO's proposal for two new bodies to promote efforts in this field. A Consultative Group on Appropriate Technology was suggested as a mechanism for mobilising resources and determining research priorities, linked with an International Appropriate Technology Unit designed to identify and focus research in areas where technological innovation would have a significant impact.

Again lacking the support of the employers and western governments, the developing countries and the workers' group declared their support for an international code of conduct on technology transfer, as proposed by UNCTAD a month previously in Nairobi. As well as recommending a timetable for drafting and adoption of this code by the end of next year, the UNCTAD meeting also agreed on the immediate setting-up of regional technological centres under the aegis of either UNCTAD or the UN Industrial

Development Organisation.

Attention was also drawn in Geneva to the damaging restrictive practices contained in a dusty old piece of international law—the Paris Convention of 1883—which defined industrial property rights for a world very different from that of today. With its regulations and techniques which would condemn the developing countries to a permanent patents and like matters often work-

ing to the detriment of developing countries, they and the workers' group called for this Convention to be "drastically revised."

In its final report, the World Employment Conference endeavoured to strike a balance between the need for technologies which will generate the maximum possible growth of employment and the avoidance of low-productivity inferior role in the world

economy. In practice, the technology choices of developing countries tend towards the capital-intensive end of the spectrum where work places will be few and far between for the one thousand million job-hunters in the year 2000. The danger is that what the report calls a "strategy of equilibrium" will be taken by most governments as an endorsement of whatever their policy now happens to be. □

## USSR

# Down Georgia way

*Wendy Barnaby, recently in the Georgian SSR, describes some of the scientific work going on there*

It takes two and a half hours to fly from Moscow to Tbilisi, the capital of the Georgian Soviet Socialist Republic. In this sunny space between the Black and Caspian Seas, the amount of human effort devoted to science depends on how successfully it—and everything else—competes with the five million Georgians' favourite occupation: the ceremonious consumption of their excellent local wines. And, as a few days spent with the Georgian Academy of Sciences shows, science is coming out of the competition extraordinarily well.

Astrophysical astronomy is concentrated in the southern republics of the Soviet Union, where the weather permits good observation. The air is especially clear on Mt Kanobili (1,700 m), 250 km from Tbilisi and only about 40 km from the Turkish border. Spread throughout the trees on the mountain top is the Academy's Abastumani Observatory, founded in 1937 as the first experimental mountain astro-physical observatory in the USSR. Work here is mainly concerned with galactic research. The resident scientists (including many women) study stars' spectra and classify them in order to examine the distribution of the stars according to their age and chemical composition. From such a distribution investigations are made of the structure of our galaxy. So far, about 100,000 stars have been classified, using a telescope whose diameter is the third largest in the world. During their last 20 years' research, the astronomers have discovered two of the 150 Supernova stars as well as about 100 emission stars.

The observatory's stellar telescope has a diameter of 125 cm and is the biggest fully automatic one in the USSR. The computer attached to it processes 1 million bits of information a second, and its memory stores 32,000

bits. Observations, which can be made on 160–170 nights a year, are mainly for determining the stars' chemical composition, physical properties and light variation. The solar telescope is used more frequently: generally on 260 days a year. Through studying the physical properties of solar flares and sun spots, the astronomers hope to be able to predict solar activity. They also do photometric and spectroscopic studies of optic radiation of the sun's atmosphere, and of radio emission at 220 MHz. The resulting data is shared under the Solar Service Programme.

Back at Tbilisi, the Underground Gravimetric Laboratory of the Academy's Institute of Geophysics is the centre of the Communist countries' cooperative efforts to study earth tides. Half burrowed into a hill overlooking the city, the laboratory is accumulating data which it is hoped will lead to the prediction of earthquakes. In tunnels leading off from basement passages, two double-component quartz extensometers composed of welded quartz tubes have been fixed so that their movements will reflect the tidal and secular deformations of the earth's crust, enabling these to be observed. Any movement is recorded photoelectrically, with a reading of 1 mm on the tape corresponding to a change of 0.1 micron on the ground. Between 1965 and 1972, the value of the recorded drift of one extensometer in the direction N 60°E was  $11.9 \times 10^{-6}$ . The value of deformation in the direction N 30°W between 1966 and 1972 was  $14.7 \times 10^{-6}$ .

Further down the passages, tiltmeters on basalt bases record changes in N-S and E-W directions. The instruments are housed in a chamber 60 m under the ground, where the diurnal temperature variation does not exceed 0.005 °C. From 1967 to 1972, the total drift in the N-S direction was 0.4 microns, and that in the E-W direction was 3.0 microns. The readings from both tiltmeters and extensometers are interpreted by the laboratory's staff as evidence of the existence of secular movements of the earth's surface. So far

there has been no success in using this knowledge to predict earthquakes: neither at this laboratory, nor at a new seismic station nearby, where equipment is being installed for the measurement of seismic waves from local and distant events. (Incidentally, the scientists at the station claimed that their research will enable them to locate and identify nuclear explosions in rock and in the Northern Hemisphere down to less than 10 kilotons. If the station were integrated into an international net, it could presumably contribute to the verification of any future comprehensive test ban treaty).

The Gravimetric Laboratory's methods are also being used to measure the earth's movements at a hydroelectric dam under construction about 50 km from Tbilisi. The plant, which will be completed in 1978, will have a capacity of 130 MW. Its artificial lake will hold 500 million cubic metres which will irrigate 45,000 hectares of land and supply drinking water to Tbilisi and its industrial area for 80–100 years.

Another of the laboratory's activities is the suppression of hail which threatens to destroy large parts of Georgia's grape crops every year. In 1974, 20,000 rockets containing silver iodide were launched from 34 automatically-synchronised firing points to save 360,000 hectares. Radar was used to detect clouds up to 15,000 metres, and results were good if the approaching front was narrow but not so good if it was broad. Because silver iodide is very toxic and expensive, research is being done into substitutes—ice with chemicals, for example.

In addition to coping with a heavy load of scientific work, a number of Academicians—including the Vice-President—represent Georgia in the Supreme Soviet of the USSR. This involves them in political activity to a degree extremely rare for active scientists in the West. Such involvement undoubtedly has its good and bad sides, but inasmuch as it contributes to the activism of science it seems a pity that Western scientists do not become more politically involved in their own societies. □



## USSR

● The State Committee of Supply (Gossnab) of the Soviet Union is holding up scientific progress, a recent *Pravda* editorial has implied. Soviet policy is, of course, committed to the idea of the scientific and technical revolution as the panacea for social and economic ills, but according to *Pravda* Gossnab persists in supplying research institutes and experimental enterprises as if they were ordinary industrial concerns.

This criticism, from the official organ of the Party, reinforces the picture presented by individual scientists of a vast bureaucracy of order-forms, in which it is virtually impossible, once a project has started, to acquire some auxiliary equipment or reagents not envisaged in the original plan. The vivid description by such unofficial sources is of a standard procedure which includes over-ordering what is definitely needed and arranging to exchange surpluses with another laboratory to provide for unforeseen requirements. The success of a laboratory, it has even been claimed, depends not so much on the abilities of its top scientists, but on the wiliness of its "Fixer" in arranging such unofficial supplies.

Although *Pravda* makes no overt reference to this self-help system, this is clearly what lies behind the statement that "in the opinion of many scientists and specialists, what is needed is a system for the immediate supply of everything found to be necessary during the course of experiments and the development of experimental models." This "would permit the acceleration of scientific progress", presumably by making allowances for serendipity and eliminating the need for a marked degree of precision. Gossnab, however, "is delaying with the creation of such a system".

The tendency to treat science as simply another branch of production is not, however, confined to Gossnab. The implementation of basic research in industry is one of the major themes of Soviet planning; considerable funds have accordingly been allocated to this end and a large number of research bases have been set up. Not all these facilities, however, are being used for their proper purpose. Thus, the Ministry of Gas Production has an excellent research establishment at Donetsk, but instead of using it for testing experimental models of new equipment, a large proportion of it has been handed over to routine production, while only a small fraction is used for the prime purpose

of research and development. Examples of proper cooperation in this field, such as that developed by the Byelorussian Academy of Sciences and its subsidiary institutes on the one hand, and the industrial enterprises of the Byelorussian Republic on the other, to form what *Pravda* describes as "a truly powerful experimental base of science", are still, it would seem, sufficiently rare to evoke glowing press reports when they occur.



Another major problem facing Soviet research is the lack of sufficient scientific instruments. Here the problem is one of demand exceeding supply, so that in spite of considerable efforts by the relevant Ministries (Instrumentation, Automation and so on) and scientific bodies from the Academy of Sciences downwards, there are still considerable shortages. One recent development has been the establishment of rental centres, from which expensive and complex equipment may be borrowed for specific experimental projects. Such centres are already in operation in Moscow and Leningrad, and the Rental Department of the Siberian Metrology Institute, now in its fourth year of operation, supplies "hundreds of scientific and industrial organisations" of the developing regions of Siberia. The collective use of equipment, whether via rental centres or "on the principle of the comradely mutual aid and cooperation of Institutes" is especially recommended. The use of computers in planning scientific supply is urged, but with the cautious proviso that computers are to be employed thriftily.

For several years now, centralised and computerised planning has been a major aim of the logistics of Soviet science. This new emphasis on economy of computer use, and the suggestion that individual institutes might profitably make mutual equipment-sharing arrangements with

other institutes, is a far cry from legalising the present system of unofficial exchanges of specially stock-piled surpluses. It does, however, indicate a growing recognition on the part of the authorities that running research and development on the same state-wide standardised pattern used for the production industries is at least problematical.

● The Soviet space programme, being based on Tsiolkovskii's concept of long-term orbital space stations as a prerequisite of any manned missions to the moon or planets, has always been greatly concerned with the medical problem of prolonged weightlessness. The death of the Soyuz 11 team upon re-entry, although finally proved to be due to a faulty hatch-seal, at first evoked considerable fears that the tragedy was due to some unsuspected side-effect of prolonged weightlessness. Accordingly, in addition to the usual programmes of astrophysical observations and the checking of equipment, the current Soyuz 21/Salyut 5 mission is paying special attention to the effects of weightlessness.

As usual, medical data—cardio-vascular and respiratory action—is being telemetered to ground control; in addition, however, the cosmonauts have on board what the TASS agency describes as a special set of scales to enable loss of body mass to be monitored. What precise form these scales take, and how they can operate in conditions of zero gravity, is not specified. A similar question is posed by another on-board experiment—the presence of a tank of guppy fish which will be periodically filmed to record their adaptation (if any) to weightlessness—since submersion in water is a standard procedure for simulating weightlessness in pre-flight training missions.

Whatever the precise significance of these experiments, however, it seems clear that Soviet plans are moving towards longer and longer missions. Increasing attention is being paid to both physical and psychological well-being—an endless-belt "running track" and a colour-slide projector for light entertainment are carried on the latest mission. A new central control panel aboard Salyut 5 gives greater facility for the cosmonauts to operate scientific equipment during experiments, reducing their dependence on ground control and preparing the way for fully autonomous space missions whether in orbit or beyond.

Vera Rich



## IN BRIEF

## ESA scientific programme

The European Space Agency is in the process of selecting its future scientific satellite and shuttle payloads. Studies of 16 possible projects were presented to European scientists at the end of last month in Paris. The astronomy and solar system working groups subsequently made recommendations and the Science Advisory Committee then listed the next scientific projects to be undertaken: cooperation on the space telescope with NASA, an out-of-ecliptic and solar stereoscopy mission and a large infrared telescope on Spacelab. Final decisions will be taken in September, but UK scientists are meeting this week to identify their main areas of interest. They may also voice their objections about where the axe has fallen among the original projects.

## Reprocessing debate continues

The social, technological and environmental problems associated with the management of radioactive nuclear waste came under the spotlight last week during an international meeting of nuclear power experts in Denver, Colorado. Sir John Hill, Chairman of the UKAEA, pointing to national and international regulatory agencies, warned against premature adoption of inadequate short term safeguards on the one hand, and unnecessary delays to nuclear expansion pending the development of a "final [reprocessing] solution" on the other. Sir John foresaw no imminent changes in UK waste management policy, so that work on vitrification processes would continue, which need not ultimately preclude incineration. Even with widespread nuclear development, he said,

radioactive waste arisings would be small compared with world mercury and arsenic stocks.

Meanwhile, amid reports that western Europe will have only about half of the reprocessing capacity it needs by 1980, work has begun on the site for British Nuclear Fuels Ltd's (BNFL) new reprocessing facility at Windscale. With construction costs estimated at £350 millions (the UK Government has just underwritten BNFL borrowing of £75 millions) the installation is expected to handle 1,000 tonnes of spent fuel a year by the mid-1980s.

A call for an outright ban on the sale of the technology for reprocessing will, however, be made at the annual conference of the British Labour Party in September. A motion urges the UK Government to consult with the group of nuclear exporting countries on the matter.

THE name of sassafras tea conjures up thoughts of early days in North America, of folk medicine, and of a tree whose leaves, charmingly enough, grow in three different shapes on the same branch—some are three-fingered, some are mitten-shaped, and some are just oval. The name is Spanish, but the Onondaga tribe in New York State called it Wah-eh-nak-kas—"smelling stick".

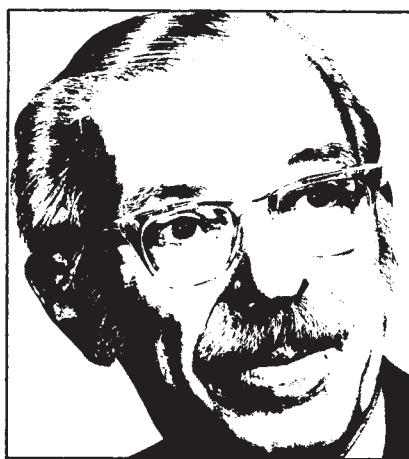
Pull up a sassafras seedling. You can peel the bark from the root, and sniff at the fragrance of the core. This spicy aroma led to its use as a flavour for root beer, which is a popular soft drink well-known to Americans and Canadians. But, alas, after many generations of people had enjoyed the natural flavour of sassafras, it turned out that safrole, the substance responsible for this, caused cancer in rats. Oil of sassafras, which contains about 80% safrole, was therefore banned as a food additive in 1960.

Manufacturers of flavoring extracts switched to methyl salicylate, present in wintergreen berries, as a substitute for oil of sassafras, and root beer continued to flow wherever soft drinks were sold in North America. The change made for a somewhat limimentous tang in the beverage, but the salicylates have a clean bill of health in the field of cancer. Also, wintergreen grows beneath sassafras trees in the Appalachian woodlands, so that there was an ecological link between the old flavour and the new.

But safrole still lurked in packages of dried sassafras root that are sold in "health food" stores to lovers of herb teas. These infusions are rumoured to be good, in a generalised

way, for what ails you. By sipping them, you get all the benefits of hot water plus whatever happens to be in dandelion heads, camomile, dried parsley, peppermint leaves, comfrey,

## Mad about safrole



THOMAS H. JUKES

rosemary (that's for remembrance), verbena, yerba maté, and, of course, sassafras.

Since these ingredients contain hundreds of compounds that have never been biologically tested for anything, herb teas are apparently considered harmless, and in addition, their consumption is hallowed by ancient custom. Unhappily for tradition, the Commissioner of Food and Drugs sternly pointed the finger "to make it clear that the prohibition on the use of safrole in food includes safrole-containing sassafras sold to make sassafras tea; effective June 10,

1976."

Comments had come from private citizens and people in the herb tea business. Several felt it was unlikely that sassafras tea is injurious to health even if safrole is carcinogenic, in one case because the tea is consumed in the spring, and in another because it had "been used for centuries without reports of carcinogenic or other toxic effects." Others noted that safrole was present in other foods such as nutmeg and mace and that hence the ban should include "all food substances containing detectable amounts of safrole."

This, of course, would be in accordance with the rules laid down by the "Delaney Amendment" in the Food Additives Law. The Commissioner replied, in effect, that he couldn't ban everything, and he had to take care of the most concentrated source of safrole first. One correspondent stated that the fat of charcoal-broiled steak contains carcinogens and that "action on sassafras should be deferred until something is done about that." No one mentioned cigarettes.

Several comments expressed various forms of anguish that a natural food, or food constituent, or ingredient, or component should be regulated, saying that this treatment should be reserved for chemical additives. Gently, the Commissioner reminded them that all food ingredients, including natural food constituents, are chemicals, also that the definition of "food" includes food additives.

The ban showed that sauce for the Red Dye No. 2 goose is also sauce for the sassafras tea gander.

# correspondence

## Plea for Argentinians

SIR,—We are writing to call the attention of your readers to the harassment many of our colleagues in Argentina are undergoing at the present time.

The Junta Militar headed by the general Jorge Videla has recently passed a law (ley de prescindibilidad, n.21.274) which allows to "release from duty" any employee of the public administration for unspecified "reasons of service", without further justification. This law has been the ground for expelling political dissenters from the universities and other scientific institutions, mainly in Buenos Aires and La Plata. The most affected institutions are, so far, the CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), the institutes depending on the Comisión Nacional de Estudios Geoheliosfísicos, and the Instituto Nacional de Tecnología Agropecuaria. Not less than 550 persons have lost their jobs: biologists, physicists, chemists, medical doctors, architects, technicians and officers, some of them after 20 years, or more, of service.

On May 7 the newspaper *La Opinión* reported that the Representative of the Junta Militar in the Ministry of Education acting as State Secretary for Science and Technology had decided to "release" 90 employees. The newspaper quoted a few names of distinguished scientists. On May 12, in *La Opinión*, Horacio Encabo (vice-Director of the Centro de Investigaciones Neurológicas Torcuato Di Tella) expressed his worries in an article emphasising the risks for the development of Argentina of a scientific policy inspired by the criterion: "quien no es occidental y cristiano es subversivo" (who is not occidental and christian is subversive). Encabo was "released from duty" five days later.

Besides protesting against these vexations there are other ways of supporting this courageous political and moral stand of our colleagues: anyone can join us by writing to those he or she may know personally or by name; informing them of his or her work by sending reprints of preprints or reports in fields of common interest; doing anything else to let them know that they are not left alone or forgotten by the world community of scientists to which they belong.

Yours faithfully,

F. ACCASCINA, G. OLIVIERI, G. TECCE, G. TOSCHI (Rome);  
G. G. ALOISI, G. MARINO, U. MAZZUCATO, G. REICHENBACH (Perugia);  
R. ANDREOLI, L. ANTOLINI, P. BARALDI, I. BARALDI, M. C. BRUNI, L. BENEDETTI, P. G. DE-BENEDETTI, R. BATISTUZZI, S. BUFFAGNI, I. M. CRAMAROSSA, G. FABBRI, A. FABRETTI, G. FINI, G. GAVIOLI, A. GUISTI, G. GRANDI, L. MENABUE, P. MIRONE, F. MOMICCHIOLI, G. PELLACANI, A. RASTELLI, E. SORAGNI, G. TOSI (Modena);  
G. BIGNAMI, A. CARPI, N. FRONTALI (Istituto Superiore Sanità, Roma);  
L. Busetto, G. L. BENDANOLI, P. BISCARINI, M. CARLOTTI, G. DI LONARDO, G. GALLONI, G. NIVELLINI, P. PALMIERI, C. STREMMENOS, F. TULLINI, A. TROMBETTI, C. ZAULI (Bologna);  
R. BARBIERI, M. CARAPEZZA, G. GIUDICE, G. LA GRUTTA, M. LEONE, F. ODDO, U. M. PALMA, M. B. PALMA-VITTORELLI, L. PAOLONI, G. SERRAVALLE (Palermo);  
E. CLEMENTI, S. PIZZINI (Istituto di Ricerche "G. Donegani" Novara);  
S. CALIFANO, E. FERRONI (Firenze);  
G. GIACOMETTI, E. VIANELLO (Padova);  
G. ALBANESE, G. CASNATI, R. FIESCHI, M. FONTANA, C. GIORI, L. OLEARI, E. SCROCCO (Parma);  
A. MONROY (Stazione Zoologica Napoli).

## Nuclear trade

SIR,—As one who objects to all nuclear explosions, I would like to comment on "Heavy Water Allegations" (June 17, page 537). While I can understand Senator Ribicoff's concern about India misusing US-supplied heavy water, I personally feel that most of those who object to India's nuclear tests are hypocritical. How else does one explain the deafening silence that greeted the revelation that Israel had at least twenty bombs in the 20–30 kiloton range? The critics of India's nuclear tests might well support Israel, using the well-worn argument that Israel is a perennially threatened state. But does any state's territorial integrity warrant a nuclear holocaust? I do not know if the US has aided Israel's nuclear programme, but I think it is reasonable to suppose that it did. If so, why does not Senator Ribicoff condemn Israeli nuclear tests?

The only way to save the world from nuclear destruction is to ensure that no country has a nuclear bomb. The present NPT cannot achieve this end because it is iniquitous. It assumes that the top five nuclear powers are the only ones which can be expected to behave responsibly. No self-respecting nation can tolerate this super-power arrogance. Those that have signed the NPT have done so under pressure. What we need today is a total elimination of nuclear arms. If this

sounds naive and unattainable then, I assert, we have to be naive and we have to attain the unattainable if we want to prevent a world-wide Hiroshima.

The cynicism which characterises all super-power pronouncements on nuclear arms must give way to an enlightened idealism. The USA and the USSR should really be talking of Strategic Arms Elimination and not of Limitation. The West has provided enlightened leadership on several issues. Is it too much to expect it to collectively lead the world back to peace and sanity? You have produced both Bertrand Russell and Ronald Reagan. Is it unrealistic to expect that you would follow the former and not the latter?

Yours faithfully,

C. S. G. PRASAD

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New Delhi 110002

## Genetic engineering

SIR,—If, as Mr Norman reports, (July 1, page 2), the NIH did indeed recommend that, for safer recombination experiments, the recombinant DNA have "less than 1 in  $10^{-8}$  chances of surviving in the natural environment", then the critics of such experiments should have even more qualms: one in  $10^{-8}$  is equivalent to 100,000,000 to 1.

Yours faithfully,

JEROME G. BUESCHER

Department of Microbiology,  
Thomas Jefferson University,  
Philadelphia, Pa.

## Human anatomy

SIR,—We were interested to see Anthony Harris's measurements of asymmetry in young women (May 6, page 10) and the response of Connolly and Dangerfield (May 20, page 188), and have found our ratios right to left of ankle 1.006, calf 1.02, biceps 1.04, wrist 1.02.

We conclude, assuming we are average females, that Anthony Harris's conclusions are correct and asymmetry is commonplace.

Yours faithfully,

JUDY WHALLEY, ENID THOMAS, HILARY LANE, MARY BROND, GAIL WESTON, SUE WILKINSON, SANDRA NIXON, PENNY ELSIP  
Department of Food Sciences,  
Polytechnic of North London,  
London, UK



# news and views

## Are earthquakes a major cause of the Chandler wobble?

from Hiroo Kanamori

WHETHER or not earthquakes are a major cause of the Earth's free Eulerian nutation (period of 14 months), widely known as the Chandler wobble, has been a controversial issue. The present consensus seems to be that the crustal deformation associated with earthquakes is much too small to explain the amplitude of the Chandler wobble. O'Connell and Dziewonski (page 259 of this issue of *Nature*), however, suggest that earthquakes represent the major factor in the wobble excitation. Since their analysis is very direct and unambiguous, it is important to examine what makes their conclusion distinct from others.

At one time, there were significant disagreements between theoretical results concerning the excitation of the Chandler wobble obtained by different investigators. This controversy, however, seems to have been completely settled, and the theory is now formulated in a rigorous framework. Observationally, the overall pattern of polar motion seems to be well established, although the data may not be good enough to allow correlation of the fine structure of the trajectory with individual earthquakes.

The physical size of great earthquakes has only recently been measured very accurately. Traditionally, the earthquake magnitude  $M_s$  (often called the Richter scale) is determined at periods of about 20 s, but it does not necessarily represent the total crustal deformation associated with earthquakes. In fact, the  $M_s$  scale is saturated at a magnitude of about 8 or when the fault length becomes about 100 km. Any earthquake whose fault length exceeds 100 km has  $M_s$  of  $8.3 \pm 0.3$  regardless of whether the fault length is 100 or 1,000 km. This does not mean that other measures of earthquake "size" such as energy or seismic moment are similarly bounded. In order to measure accurately the physical size of great earthquakes, precise measurements of long-period seismic waves are necessary. The amount of the overall crustal deformation is best expressed by the seismic moment  $M_0$  which is proportional to

the product of the fault area and the average displacement on the fault. With the recent progress in long-period seismology, the seismic moment has been determined for many great earthquakes. The fault geometry of great earthquakes is also important. With the advent of plate tectonics, it is reasonable to assume a fault geometry which is consistent with the contemporary plate motion.

In these circumstances, the paper by O'Connell and Dziewonski represents a very timely contribution. Most discussions in the past have dealt with either statistical correlations or correlations between an individual break in polar motion and a particular earthquake. The statistical arguments are somewhat indirect, and the arguments on individual events are hampered by the poor quality of the data. In order to circumvent these shortcomings, O'Connell and Dziewonski used geometries of great earthquakes predicted by plate

tectonics, and computed the cumulative effects of earthquakes on polar motion rather than the effect of individual events.

Since the seismic moment  $M_0$  is not known for most old earthquakes, O'Connell and Dziewonski estimated it by using the relation  $\log M_0 = 8.8 + 2.5 M_s$  which is obtained from the work of Chinnery and North (*Science*, **190**, 1197-1198; 1975). The  $M_s$  scale used in the above formula is based on Gutenberg and Richter (*Seismicity of the Earth*, Princeton University Press, 1954) and is very close to what is called the 20 s surface-wave magnitude. On the other hand, O'Connell and Dziewonski used the magnitude scale adopted in Duda's catalogue (*Tectonophysics*, **2**, 409-452; 1965) to estimate  $M_0$ . The magnitude scale used in Duda's catalogue is, however, different from Gutenberg-Richter's  $M_s$ ; the former is systematically larger than the latter, a fact which is not widely recognised.

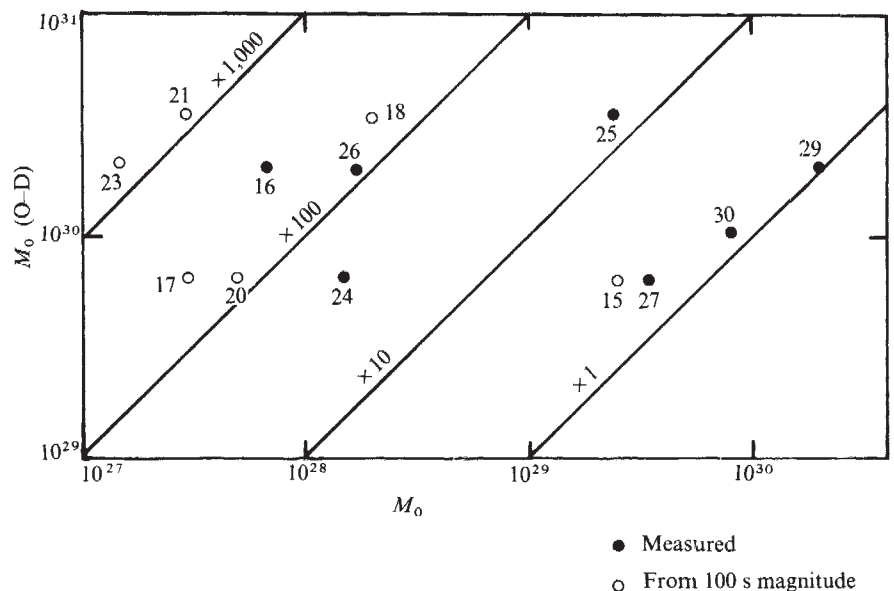


Fig. 1 Measured seismic moment  $M_0$  versus seismic moment  $M_0$  (O-D) assigned by O'Connell and Dziewonski for thirteen earthquakes. Closed circles indicate earthquakes for which  $M_0$  is directly determined (from Kanamori and Anderson, *Bull. Seismol. Soc. Am.*, **65**, 1073-1095; 1975; Ben-Menahem, Aboodi, and

Schild, *Phys. Earth planet. Int.*, **9**, 265-289; 1974), and open circles are for earthquakes for which  $M_0$  is estimated from the 100 s magnitude (Brune, and Engen, *Bull. Seismol. Soc. Am.*, **59**, 923-933; 1969). Numbers refer to those in Table 1 of O'Connell and Dziewonski.

For shallow ( $H \leq 60$  km) earthquakes for the period from 1905 to 1952 listed in Table 1 by O'Connell and Dziewonski, the average difference between the two scales is 0.3. Thus, the use of Duda's values in the above  $M_0$  versus  $M_s$  relation results in an over-estimate of  $M_0$  by a factor of 5.6. Since the polar shift is linear with the seismic moment, this difference would mean that the effect on the Chandler wobble computed by O'Connell and Dziewonski is, on the average, 5.6 times too large. However, it is more important to note that because of the saturation of the  $M_s$  scale beyond 8, any  $M_0$  versus  $M_s$  relation is useless for earthquakes of  $M_s \geq 8$ . For these large earthquakes direct determinations of  $M_0$  are essential. Fortunately, for seven earthquakes listed in Table 1 of O'Connell and Dziewonski, a direct determination of  $M_0$  has been made, and for six other earthquakes the 100 s magnitude (from which  $M_0$  can be estimated fairly accurately) has been determined. In

Fig. 1, these seismic moments are compared with those estimated by O'Connell and Dziewonski from Duda's catalogue through the  $M_0$  versus  $M_s$  relation. For earthquakes with very large rupture dimensions such as the Chilean (No. 29), the Alaskan (No. 30) and the Kamchatka (No. 27) earthquakes, the agreement is very good, but for many earthquakes with smaller rupture dimensions,  $M_0$  seems to be overestimated by more than two orders of magnitude. For most events before 1923 no direct determination of  $M_0$  has been made, but it seems almost certain from the above comparison that  $M_0$  is overestimated in a similar manner.

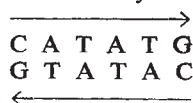
O'Connell and Dziewonski, in fact, considered most of these problems carefully in their article, but in view of the good accuracy of moment determinations even for relatively old earthquakes it appears that the direct effect of earthquakes is about one order of magnitude too small to explain the

Chandler wobble. But as they suggest, it is possible that earthquakes accompany very large aseismic deformation which has not been detected by existing seismological instruments. Then the fact that O'Connell and Dziewonski could explain the temporal behaviour of the Chandler wobble amplitude by using the mechanisms of earthquakes derived from plate motion may suggest that accelerated, but aseismic, plate motions are associated with great earthquakes (Anderson, *Science*, **182**, 49–50; 1974). Although such aseismic deformation has been suggested from several independent observations (of tsunami earthquakes, slow pre- and post-seismic deformations, disparity between plate motion and seismic slip rate for example), the details are at present unknown. With world-wide deployment of high-quality ultra-long period instrumentation (see Agnew *et al.*, *EOS*, **57**, 180–188; 1976), it is hoped that the nature of such aseismic deformation will be revealed in the near future.  $\square$

## Long palindromes in eukaryotic DNA

from T. Cavalier-Smith

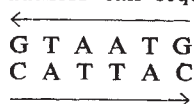
ONE of the most fascinating differences in the organisation of DNA in eukaryotic cells (those with a distinct nucleus) from that in prokaryotes (bacteria and blue-green algae) is the abundant occurrence—in higher eukaryotes at least—of long palindromes. When applied to nucleotide sequences in double-stranded DNA the word 'palindrome' usually means regions of DNA with an axis of two-fold rotational symmetry which would be read the same in both directions by the cell's transcription machinery:



would for example, be transcribed to give an RNA molecule with the sequence C A U A U G whichever end

the RNA polymerase started, because the polarity of the transcript (shown by the arrow) is always antiparallel to that of the template strand.

(A few authors call sequences like



"palindromes", but though when no account is taken of polarity each individual strand appears palindromic, the sequence does not show two-fold symmetry, and if it were read in both directions by RNA polymerase two quite different RNA sequences would

be made—its biological meaning is quite different when read backwards. Here I use 'palindrome' only for sequences with two-fold symmetry.)

Since palindromes are inverted repeats they can be easily identified because the two strands are self complementary. If denatured to separate the strands and then returned to re-naturing conditions each strand will rapidly fold back on itself to form a double-stranded hairpin loop:



These can be separated from non-palindromic DNA because they bind strongly to hydroxylapatite. Wilson and Thomas (*J. molec. Biol.*, **84**, 115; 1974) found numerous almost perfect palindromes in *Drosophila*, *Xenopus* and human DNA. Usually 300–1,200 nucleotide pairs long, they form several percent of the total DNA and like non-inverted medium repetitive sequences, are widely dispersed in the DNA and not clustered in some special fraction. Such long palindromes are absent in prokaryotes and may be of profound significance for the organisation of eukaryote chromosomes. In searching for explanations one must bear in mind that not all eukaryote palindromes are perfect. Many are not or have impaired terminal loops (Schmid *et al.*, *Cell*, **5**, 159; 1975; Perlman *et al.*, *Cell*, **8**, 33; 1976).

Palindromes could have many func-

tions. Short ones can serve as recognition sites on the DNA for proteins which themselves have two-fold rotational symmetry. Such short palindromes do exist in prokaryotes: the recognition sites for the *lac* repressor and several bacterial restriction nucleases are palindromes, but these are too short to be detected by hydroxylapatite which in conditions usually used only binds double strands of over 50 base pairs. Another suggestion is that palindromes form pairs of lateral hairpin loops in the DNA which could be important for recombination or other functions. (Sobell, *Prog. Nucl. Acid Res. molec. Biol.*, **13**, 153; 1973). Such paired loops have not been found in isolated DNA, though they can be generated *in vitro* (Karrer and Gall, *J. molec. Biol.*, **104**, 421; 1976). A looped secondary structure (like that of tRNA on a larger scale) would be expected in RNA transcripts of palindromic DNA and has been found in nuclear RNA, including both heterogeneous nuclear RNA and low molecular weight nuclear RNA, though not in cytoplasmic mRNA; however, the length of these loops (which could have an important role in regulation of transcription or RNA processing or transport) is generally shorter than the palindromes seen in the DNA.

Recent studies in *Xenopus* (Perlman *et al.*, *op cit.*) argue against a specific control function for long palindromes. It appears from renaturation kinetic



studies that the unique DNA attached to isolated palindromic DNA can include all unique sequences in the genome, and not just a small specific subfraction as would be expected if the palindromes were always attached to the same adjacent sequences. Perlman *et al.*'s interpretation is that the 100,000 palindromes of *Xenopus* are inserted at different sites in different copies of the genome, and that at any one time one tenth or fewer of the palindromic insertion sites (which must occur at average intervals of 2,500 base pairs or less) are occupied. If correct, this implies a more fluid model for the eukaryote genome than is traditional. One would have to postulate either that palindromes are continually being generated or destroyed or are being swapped from one chromosome site to another, probably even during the lifetime of a single individual. Schmid and Deininger (*Cell*, **6**, 345; 1975) have shown that palindromes are randomly positioned with respect to unique and middle repetitive sequences in human DNA.

In 1974 I suggested a mechanism for the replication of the ends (telomeres) of linear chromosomes which requires terminal palindromes (not necessarily very long ones) and an endonuclease specific for the palindromic sequence (*Nature*, **250**, 467). There are indications that a number of viruses, notably adeno-associated virus (Strauss *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **73**, 742; 1976) may replicate in this way; moreover, as predicted, the ends of the gene-sized minichromosomes of hypotrich ciliate macronuclei are palindromic and probably generated by palindrome specific endonucleases (Wesley, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 678; 1975). Now Karrer and Gall (*J. molec. Biol.*, **104**, 421; 1976) and Engberg *et al.* (*J. molec. Biol.*, **104**, 455; 1976) have both shown that in *Tetrahymena* macronuclei, where in contrast to hypotrich ciliates the bulk of the DNA is not cleaved into gene-sized pieces, the rDNA exists as numerous small linear DNA molecules each of which is a perfect palindrome over 20,000 nucleotide pairs long. This rDNA is amplified 20 times in total amount relative to the rest of the macronuclear DNA, but each molecule contains only two (inverted) copies of each of the two RNA genes.

If, as Karrer and Gall postulate, these long palindromes also have short identical inverted repeats at each end, they could replicate either according to my model or by way of a circular intermediate. Terminal 'minipalindromes' would make it very easy to explain their formation during amplification by failure of the sequence specific nuclease to nick just one end. The study of this process could have

far reaching implications for the understanding of eukaryote chromosome organisation. It is in fact very tempting to suggest that all eukaryote chromosomes, and not merely those of hypotrich ciliates, consist of a linear array of potentially independent replicative units terminated by short palindromic sequences.

To end on an even more speculative note, Sparrow and Naumann (*Science*, **192**, 524; 1976) review evidence that the doubling of whole chromosomes may be a major cause of the evolutionary increase in genome size; they suggest this could occur in linear chromosomes by a slight modification of my palindromic telomere replication mechanism suggested by Bateman (*Nature* **253**, 379; 1975) so as to generate a double length chromosome that would (until the genes inevitably diverged during evolution) be a single palindrome like that of the *Tetrahymena* rDNA giant palindrome only far longer. □

## Expression of a eukaryotic gene in *Escherichia coli*

from John Atkins

THE development of the technology of cloning DNA fragments from diverse sources in *E. coli* and potentially other organisms, by linking them to a plasmid or phage replicon has provoked much discussion. The possible expression of eukaryotic DNA to produce functional proteins when cloned in *E. coli* has been a key question. A positive answer would greatly facilitate the isolation of a substantial number of eukaryotic genes by enabling their protein products to be detected by complementation of well-studied prokaryotic mutants. Currently the chromosomal eukaryotic genes, which have been identified on cloned segments, are limited to those whose mRNA copies are a large proportion of the total mRNA, generally of some specialised cell type or after appropriate induction, and so can be isolated in relatively pure form. The ribosomal RNA genes also belong to this category (see *News and Views*, *Nature*, **259**, 173; 1976). Thus an assay based on complementation of auxotrophs would provide a significant extension of the range of genes which could be identified and so studied. Another main reason for the interest in whether eukaryotic genes can be expressed at the protein level, using the existing vectors, is industrial. Will the vectors which have been developed for *E. coli* hosts enable certain valuable eukaryotic proteins to

be produced in organisms such as *E. coli* when grown in fermenters?

A number of different groups have started experiments to answer this question about the expression of eukaryotic genes in *E. coli*, but credit is due to Struhl, Cameron and Davis who describe a careful genetic analysis of the first successful experiments in the current issue of the *Proceedings of the National Academy of Sciences* (**73**, 1471-1475; 1976). As the cloning vector this Stanford-based group used bacteriophage lambda. One of the features of certain  $\lambda$  vectors is that transcription originating at one of the powerful  $\lambda$  promoters ( $P_L$ ) continues into the heterologous cloned DNA and does not terminate at normal (that is rho) termination sites. The Stanford group did not have to utilise this feature of  $\lambda$ , however, as they found that transcription was initiated within the yeast fragment. Whether transcription originated at a sequence fortuitously recognised by *E. coli* RNA polymerase (compare cloned mouse mitochondrial DNA, Chang, Larsman, Clayton and Cohen, *Cell*, **6**, 231; 1975) or at the correct yeast promoter, is unknown.

The two *E. coli* mutants used for the complementation tests were histidine auxotrophs both with non-revertible non-suppressible mutations in the *hisB* gene such that imidazole glycerol phosphate (IGP) dehydratase activity was not present, although the other enzyme activity, histidinol phosphatase, coded by the same gene was retained (the complementation patterns in the *hisB* gene and relationship to the subunit composition of the two enzyme activities are discussed by Loper *et al.*, *Brookhaven Symp.*, **17**, 15; 1964). Struhl *et al.* isolated  $\lambda$  phages carrying yeast DNA which complemented both *his* mutants lacking IGP dehydratase although they did not complement single site mutants lacking histidinol phosphatase. This is reassuring as the yeast genes coding for the two enzyme activities are widely separated on the yeast genome. Davis and coworkers next made  $^{32}\text{P}$ -labelled complementary RNA *in vitro* using *E. coli* RNA polymerase and DNA from the  $\lambda$  carrying the yeast histidine gene as a template. This RNA was then hybridised to separated fragments derived from an independently prepared yeast DNA sample, and the DNA fragment which gave positive hybridisation was the same size as the cloned yeast fragment from which the complementary RNA was synthesised. As the authors point out, this argues against any contaminant phage being responsible for the complementation. Struhl *et al.* have not yet characterised the enzyme produced, nor the DNA fragment in detail, and any real measure of the efficiency of translation also awaits study. This latter

aspect is of interest as one of the very few reports from analogous cell-free translation studies gave a low efficiency of translation (Crawford and Gesteland, *J. molec. Biol.*, **74**, 627; 1973) and whether the efficiency is greater *in vivo* (in relation to other *in vivo* translation), or if it is very message dependent, is unknown. Consequently, any generalisations from the present study should await the results of complementation studies done with a considerable number of different auxotrophs.

The presentation at the recent Miles Symposium by J. Carbon and colleagues suggest that such reports will soon be forthcoming. □

## More rapidly growing manganese

from Peter J. Smith

Two years ago Scott *et al.* (*Geophys. Res. Lett.*, **1**, 355; 1974) reported the discovery of an unusual manganese oxide crust about 5 km from the axis of the mid-Atlantic ridge at 26°N. The sample in question, a 4.2 cm thick deposit of birnessite with a trace of todorokite, had an abnormally low iron concentration (compared with typical ferromanganese nodules and crusts), was depleted in  $^{230}\text{Th}$  and  $^{231}\text{Pa}$  and contained unexpectedly small amounts of Cu, Ni and Co. Its most startling characteristic, however, was its rapid growth rate. Whereas ferromanganese nodules and crusts grow on the ocean floor at typical rates of 0.1–1.0 cm per million years, the new sample had apparently grown at a rate of 13.0–25.0 cm per million years.

On the basis of all the evidence Scott and his colleagues concluded that their newly-discovered crust was a product of hydrothermal activity along an active ridge crest and had not, as 'normal' ferromanganese crust, precipitated from seawater. At that time the importance of hydrothermal processes at ridges, though not unrecognised (for example, Lister, *Eos*, **55**, 740; 1974 and others had recently drawn attention to it), was not widely appreciated. Today, however, the situation is rather different. Hydrothermal activity is increasingly commonly acknowledged to play a major part in heat transfer at oceanic ridges, probably explaining both the great variability of axial heat flow and the low average axial heat flow observed compared with that predicted from cooling lithosphere models. It is probably also closely involved with the formation of metal-rich sediments at ridge crests.

It is in this changing context that Moore and Vogt (*Earth planet. Sci. Lett.*, **29**, 349; 1976) now report the discovery of two more manganese oxide crusts (A and B), similar to that of

Scott and his coworkers but from the vicinity of the Galapagos spreading axis. Sample A is a 2–4 cm thick deposit recovered from an almost sediment-free wall of an axial valley of the spreading centre where the basement age estimated from magnetic anomalies is 0.3 Myr. It consists of a grey banded zone comprising mixed todorokite and birnessite covering a darker friable zone of todorokite. Assuming this sample to have remained a chemically closed system, its maximum age obtained from the  $^{230}\text{Th}/^{234}\text{U}$  ratio is 6,000 years at a depth of 9–13 mm within the crust and 9,000 years at 6–9 mm. On this basis the growth rate for the outer 1 cm was therefore 100–200 cm per million years, or 100–2,000 times greater than the rate for seawater-precipitated deposits.

Compared with typical ocean floor ferromanganese deposits, sample A has very low Fe/Mn and  $^{232}\text{Th}/^{238}\text{U}$  ratios, a higher manganese concentration and lower transition metal concentrations. These properties, combined with the rapid growth rate, strongly support the idea of formation by ridge hydrothermal activity in an area where the supply of manganese is enhanced. Indeed, circumstances suggest that hydrothermal activity may be more vigorous here than is usual along spreading axes, for the recovery site is close to the position of the proposed Galapagos hot spot. Be that as it may, the chief competing explanation—that the extra manganese is remobilised from a sedimentary source—would seem to be ruled out by the obvious lack of sediment at the recovery site and a direct association of the crust with igneous rocks such as tholeiitic basalts and, unusually, soda rhyolite.

Sample B, a 4–6 cm thick crust recovered further from the ridge axis at a point where the basement age is 2.4 Myr, is more complex, less amenable to study but in some ways more interesting than sample A. It has a 4–6 cm core of grey todorokite and birnessite which is chemically similar to sample A and thus believed likewise to be of hydrothermal origin. But, by contrast, the core is covered with a 3 mm thick layer of darker material indistinguishable from seawater-precipitated manganese deposits.

Chemical analysis shows that this sample could not have remained a closed system. No ages can therefore be calculated from the  $^{230}\text{Th}/^{234}\text{U}$  ratio, although given the basement age the growth rate must have been at least 2.5 cm per million years. Assuming average precipitation growth rates, the 3 mm covering would then have taken about 1 million years to form around the core. The implication is that the core formed hydrothermally whilst the sample was close to the spreading axis,

but acquired a seawater-precipitated coating more slowly as it was taken out of the hydrothermal zone by seafloor spreading.

Taken together, these two samples provide further support for the existence of hydrothermal processes at ridge crests. Why such activity should lead to extreme fractionation of iron and manganese, and thus to deposits abnormally rich in manganese, is another question, however. Moore and Vogt support the view that the iron was removed from the hydrothermal solutions before they debouched into the seawater. Bonatti *et al.* (*Econ. Geol.*, **67**, 717; 1972), following Krauskopf, noted, for example, that iron should precipitate from a hydrothermal fluid before manganese as sulphides or oxides, thereby gradually lowering the Fe/Mn ratio in the remaining fluid. Clearly this process could produce a solution depleted in iron so that at debouchment the manganese would be rapidly oxidised and precipitated. But this explanation is almost certainly incomplete—and there are other possibilities. The full details and significance of hydrothermal activity at ridge crests have yet to be worked out.

## Borrowed polypeptides in Q $\beta$ replicase

from Pamela Hamlyn

THE enzyme which replicates the genetic material of the single-stranded RNA phage Q $\beta$ —Q $\beta$  replicase—has many interesting properties, not least its specificity. The enzyme recognises, and begins initiation, at the oligonucleotide CCCA found at the 3' end of both the plus and minus strands (it is a two step replication). The first residue to be incorporated is a G; the A is not copied. *In vitro* the enzyme will replicate poly(C) and C-containing copolymers which suggests it is not too choosy, perhaps only requiring some Cs at the 3' terminus. But it will not replicate the RNA of other similar phages which also end with CCCA. Weissman has proposed—and there is some supporting evidence—that as well as the terminal Cs the enzyme also recognises another region within the RNA molecule and it is the spatial relationship between these regions that acts as a signal for initiation (Weissman *et al.*, *A. Rev. Biochem.*, **42**, 303; 1973).

Another notable feature of Q $\beta$  replicase is that three of the four subunits comprising the enzyme are polypeptides 'borrowed' from the host—*E. coli*. One is the ribosomal protein S1 which the host uses for the correct



recognition and binding of mRNA. The other two requisitioned polypeptides are also involved in protein synthesis in the host; they are the polypeptide elongation factors (EF) Tu and Ts. Their known function in protein synthesis could be very neatly fitted with possible functions in the Q $\beta$  replicase. EF-Tu is able to bind GTP which is the only nucleotide with which Q $\beta$  replicase can initiate replication. EF-Ts can remove GDP from EF-Tu in a catalytic exchange reaction. In addition, EF-Tu is able to bind aminoacyl-tRNA and, since Q $\beta$  RNA and tRNA both have CCA at their 3' end, the suggestion was readily made that EF-Tu in the replicase was involved in enzyme-template binding.

The method used to test these hypotheses was to alter the EF-Tu and EF-Ts subunits so that they are no longer effective in supporting protein synthesis, and then to reconstitute Q $\beta$  replicase with these modified subunits and test for enzymatic activity with synthetic and natural templates. Brown and Blumenthal have performed these experiments with unexpected results. (Brown and Blumenthal, *J. biol. Chem.*, **251**, 2749; 1976; and *Proc. natn. Acad. Sci. U.S.A.*, **73**, 1131; 1976). Using chemical modifiers, they have altered the aminoacyl tRNA binding site of the enzyme subunit which is EF-Tu and shown that, although it now no longer supports protein synthesis, Q $\beta$  replicase reconstituted with it is active—in fact its specific activity is very similar to the native enzyme. To investigate the GTP binding site on EF-Tu they used kirromycin, an antibiotic that inhibits protein synthesis by altering the GTP binding properties of EF-Tu but has no effect on the aminoacyl-tRNA binding site. Low levels of kirromycin which effectively inhibit protein synthesis do not affect the replicase activity, suggesting that in replicase EF-Tu does not function by binding GTP. Further careful experiments using reconstituted replicase revealed that kirromycin acted on the enzyme by preventing the formation of an EF-Tu-Ts complex. The authors think that it is as a complex that these two subunits function in native replicase. Support for this is provided by the experiments described in their paper in the *Proceedings of the National Academy of Sciences*. EF-Tu and EF-Ts have been chemically 'crosslinked' (joined covalently) and this complex used to reconstitute the enzyme. Although in this state the elongation factors cannot perform their function in protein synthesis, they do not at all inhibit the activity of the reconstituted enzyme.

Since the replicase is not utilising the known abilities of EF-Tu and EF-Ts perhaps they perform other functions

in the host, as yet undiscovered by scientists but recognised by Q $\beta$  as useful. More likely the authors think, is that the two subunits have some structural role, perhaps holding subunit II, the one coded for by Q $\beta$ , in its active configuration. The enzyme has the ability to unfold its template RNA as it copies it and to keep the two complementary strands apart. Perhaps EF-Tu-Ts could be involved. □

## Zinc in enzymes

from Peter J. Sadler

WHEN cultured in normal conditions, yeast uses a zinc metalloenzyme, alcohol dehydrogenase (YADH), during the final stage of the fermentation process, for the conversion of acetaldehyde to ethanol. The role of zinc in the enzyme is intriguing. If Co<sup>2+</sup> is added to the culture medium it becomes incorporated into the enzyme, and green (but still active) enzyme crystals instead of colourless (Zn) ones (Curdell and Iwatsubo, *FEBS Lett.*, **1**, 133; 1968) can be isolated. Furthermore, if Zn<sup>2+</sup> is excluded from the growth medium and Mn<sup>2+</sup> is added, an active Mn<sup>2+</sup> enzyme is isolated (Coleman and Weiner, *Biochemistry*, **12**, 3466; 1973).

The natural yeast enzyme has a molecular weight of 140,000 and contains four zinc ions. Their role is thought to be similar to the four zinc ions in the mammalian liver enzyme (molecular weight 80,000) which converts ingested alcohol to acetaldehyde. This enzyme (LADH) has recently been the subject of X-ray crystallographic analyses at 2.4 Å resolution (Eklund *et al.*, *J. molec. Biol.*, **102**, 27; 1976) and detailed metal exchange studies in solution (Stykowski and Vallee, *Proc. natn. Acad. Sci. U.S.A.*, **73**, 344; 1976). A close look at these results reveals the conditions in which metal ions can be swapped and activity retained.

Early attempts to remove zinc from LADH and to reconstitute it with other metal ions met with difficulty. Enzyme activity decreases as zinc is removed, but slow conformational changes occur through oxidation of formerly stable SH groups to S-S bonds (Oppenheimer, Green and McKay, *Archs Biochem. Biophys.*, **119**, 552; 1967).

But successful exchange reactions can be performed under an N<sub>2</sub> atmosphere. The zinc atoms behave in pairs: Zn<sub>2</sub> (Zn<sub>2</sub>) representing non-catalytic (catalytic) ions. The pairs exchange at different rates. For example, Zn<sub>2</sub> (Zn<sub>2</sub>) dialysed against radioactive <sup>65</sup>Zn<sub>2</sub> (Zn<sub>2</sub>) initially becomes <sup>65</sup>Zn<sub>2</sub> (Zn<sub>2</sub>) and finally <sup>65</sup>Zn<sub>2</sub> (<sup>65</sup>Zn<sub>2</sub>). Similarly, <sup>65</sup>Zn<sub>2</sub> (Zn<sub>2</sub>) with Co<sup>2+</sup> added becomes Co<sub>2</sub> (Zn<sub>2</sub>) after 12 h (4 °C, pH 5.5), and Co<sub>2</sub> (Co<sub>2</sub>) after 120 h. But Co<sub>4</sub> (Zn<sub>2</sub>) has an identical activity to Zn<sub>2</sub> (Zn<sub>2</sub>), whereas Co<sub>2</sub> (Co<sub>2</sub>) is only

64% as active. Hence the term catalytic to describe the second pair of zinc ions.

The crystallographic analysis shows the enzyme to be a dimer of two identical subunits, each having two zinc binding sites and one coenzyme (NAD<sup>+</sup>) site. As predicted by earlier spectroscopic studies (Foster, Hill and Williams, *Biochem. Soc. Symp.*, **31**, 187; 1970) on YADH, the zinc coordination sites are (distorted) tetrahedral and include cysteine (sulphur) ligands: four cysteines for the non-catalytic zinc, and two plus one histidine (N) and one water molecule for the catalytic zinc. The latter, which is the faster ion in exchange, is situated at the bottom of a 20 Å pocket, whereas the non-catalytic zinc is in a cleft on the enzyme's surface.

An alternative differentiation of the zinc atoms involves binding 1,10-phenanthroline (OP). OP inhibits reversibly Zn<sub>2</sub> (Zn<sub>2</sub>) or Co<sub>2</sub> (Zn<sub>2</sub>) by binding at catalytic zinc (H<sub>2</sub>O displacement) without removing them. OP irreversibly inhibits Co<sub>2</sub> (Co<sub>2</sub>) by removing the catalytic cobalt ions. A difference in behaviour of Co and Zn is notable here.

A near-tetrahedral zinc with a water ligand is now becoming familiar at zinc sites in metalloenzymes: carbonic anhydrase (3 His, H<sub>2</sub>O), carboxypeptidase (2 His, Glu, H<sub>2</sub>O), thermolysin (2 His, Glu, H<sub>2</sub>O). Woolley (*Nature*, **258**, 677; 1975) has recently discussed the enhanced reactivity of such water ligands. This may vary with the fractional change placed on Zn by the other ligands, and may account for the exceptional catalytic efficiency of carbonic anhydrase which has one of the largest known turnover numbers, 600,000 s<sup>-1</sup>.

The non-catalytic zinc or cobalt ions in LADH may simply have structural roles, but this coordination site is strikingly similar to the iron site in rubredoxin and bacterial ferredoxins, and a sequence spacing of coordinated cysteines, X, X + 3 is a regular occurrence. Substitution of the non-catalytic zinc with iron should not only produce an enzyme with interesting properties, but also perhaps with evolutionary significance! □



### A hundred years ago

VIOLENT shocks of earthquake were felt again on the 5th inst. at Corinth and the surrounding district. The direction of the motion was east to west. Shortly after noon on Monday an earthquake occurred in Vienna. Three violent shocks, lasting two seconds, were felt. A panic ensued. Several houses are damaged, and a portion of the old walls has been split.  
from *Nature*, **14**, July 20, 260; 1876

# articles

## Excitation of the Chandler wobble by large earthquakes

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*Superposition of the polar shifts computed for 234 large earthquakes ( $M_s \geq 7.8$ ) that occurred between the years 1901 and 1970 yields a synthetic curve that closely resembles the observed pattern of variations of the Chandler wobble during the same period. We conclude that earthquakes represent the major factor in the wobble excitation.*

SINCE its discovery in 1891, the Eulerian free nutation of the Earth, popularly called the Chandler wobble, has been of considerable interest to astronomers, meteorologists, oceanographers and solid earth geophysicists. Throughout this time the mechanisms exciting the wobble have remained elusive, and although atmospheric motions, core-mantle interactions and earthquakes have all been considered, none of these has been either clearly identified as the principal source of excitation or entirely rejected as a partial contributor.

The period of the wobble depends on the Earth's oblateness and its elastic properties, and the observed period is reasonably well accounted for by current Earth models, with the greatest uncertainty coming from lack of exact knowledge of the effect of the oceans<sup>1</sup>. The observed variation of amplitude of the wobble has not been satisfactorily explained, although several different mechanisms have been proposed. These variations must be caused by damping and the excitation of the wobble. These two mechanisms cannot be studied independently, of course, since the excitation needed to account for the observed wobble depends on the magnitude of damping.

None of the estimates of the frequency or damping of the wobble have explicitly considered the mechanisms exciting the wobble, and it is conceivable that particular excitations could introduce considerable bias into estimates of the intrinsic frequency and damping of the wobble. For this reason it is advantageous to calculate the excitation from specific mechanisms to remove any such biases, before interpreting the results in terms of the intrinsic properties of the Earth.

In this study we consider the cumulative effect of major earthquakes that occurred between 1901 and 1970 as a source of excitation of the wobble. Previous investigations of the role of earthquakes in exciting the wobble have yielded no clear conclusions. Although Munk and MacDonald<sup>2</sup>, among others, had concluded that displacements associated with earthquakes were too small to excite the wobble, Smylie and Mansinha<sup>3</sup> presented evidence for a correlation between times of occurrence of major earthquakes and changes in the direction of polar motion. This suggested that earthquakes could be responsible for a sizeable fraction of the excitation and revived interest in

the subject which has become one of the more controversial topics in the geophysical literature.

Subsequent studies<sup>4,5</sup> concentrated on the correlation between individual earthquakes and short term changes in polar motion. Because of the considerable uncertainty in the exact location of the pole at any time, a definite correlation between polar motion and earthquakes could not be firmly established<sup>6,7</sup>. In addition, Dahlen<sup>8</sup> concluded that earthquakes could not account for the observed amplitude of the wobble; his result was based on the calculated pole shifts for the 1960 Chilean and 1964 Alaskan earthquakes and a Monte Carlo simulation of seismicity averaged over the whole Earth.

Nevertheless, there is still highly suggestive evidence of a correlation between global seismicity and changes in the amplitude of the wobble over the period of the past 70 yr (refs 9–11). This suggests that the cumulative effect of earthquakes may account for the long term variations in the amplitude of the wobble, even though the effect of any individual earthquake is too small to be detected above the noise in the data on polar motion. To investigate this possibility we calculated the polar motion expected from major earthquakes over the past 70 yr and compared it with actual polar motion data over the same period. This requires specification of the time, location and source parameters of the earthquakes, as well as the calculation of the change of the Earth's inertia tensor caused by an earthquake.

### The synthetic Chandler wobble

The evaluation of the change in the Earth's inertia tensor involves calculation of the static displacement field resulting from an earthquake. Previous workers have solved this problem directly<sup>8,12–14</sup>, but some of the numerical results differed by an order of magnitude. Here we make use of the representation of the static displacement field by a linear superposition of normal modes<sup>15</sup>; only the normal modes of degree 0 and 2 contribute to changes in the moment of inertia. The advantage of this approach is that it has been empirically tested; all the modes that significantly contribute to the change in the inertia tensor have been observed (modes  ${}_0S_0$ ,  ${}_0S_2$  and  ${}_1S_2$  account for more than 95% of the effect) and their excitation and eigenfrequencies can be explained by realistic models of the Earth's structure and the seismic source mechanism. In addition, this independent calculation resolves the differences among the numerical results obtained by other workers.

The change in the inertia tensor  $C_{ij}$  caused by an earthquake with an equivalent source moment tensor  $M_{kl}$  can be written

$$\Delta C_{ij} = D_{ijkl} M_{kl}$$



where  $D_{ijkl}$  is a fourth rank tensor whose components depend on 6 canonical functions of the model of the Earth evaluated at the source depth, and the latitude and longitude of the epicentre. The components of  $D_{ijkl}$  and canonical functions for both a uniform Earth model and a realistic Earth model PEM-C (ref. 16) are given in Dziewonski and O'Connell<sup>17</sup>. The expression of the inertia change in terms of the complete moment tensor  $M_{kl}$  permits consideration of source mechanisms more general than a double couple<sup>18,19</sup>.

The changes of the inertia tensor caused by the 1960 Chilean and 1964 Alaskan earthquakes calculated using the normal mode approach agree well with those calculated by Dahlen<sup>8</sup>, so long as similar Earth models are used. Substantial differences can result from using Earth models that differ at the source depth, and a uniform Earth model gives noticeably different results from a realistic Earth model<sup>17</sup>.

To specify the source moment tensor for an earthquake one needs to know the orientation of the fault plane and direction of the slip vector (for lack of better evidence, at this time, we shall use the traditional double couple model) as well as the scalar seismic moment. Most large earthquakes occur at plate boundaries and the source mechanisms that have been determined for several earthquakes are remarkably consistent with current plate tectonic models; for example refs 20–23. This suggests that one may reliably estimate the source parameters of large earthquakes on plate boundaries from knowledge of the type of a plate boundary and relative motion between the plates.

The earthquakes with magnitudes  $M_s \geq 7.8$  used to calculate the excitation of the Chandler wobble are taken from the catalogue compiled by Duda<sup>24</sup>, primarily based on the study of Gutenberg and Richter<sup>25</sup>, and supplemented by information from the *Bulletin of the Seismological Society of America* for the years 1965–1970. Since it is the seismic moment  $M_0$  that determines the static displacement, we have used an approximate empirical relation to determine the moments from the reported magnitudes. Figure 1, after Chinnery and North<sup>26</sup>, shows the relation between  $M_0$  and  $M_s$  for a number of earthquakes for which both have been independently determined.

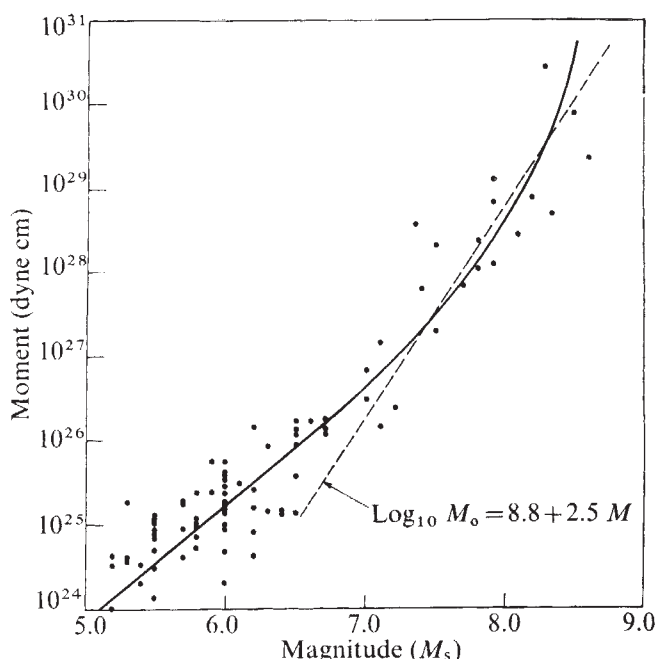


Fig. 1 Relation between seismic moment  $M_0$  and magnitude  $M_s$  after Chinnery and North<sup>26</sup>. The dashed line gives the relation used to calculate the pole shifts from earthquakes.

The solid line is the relation between moment and magnitude proposed by Chinnery and North. The dashed line shows the relation we have used to determine the moments; it obviously is a reasonable approximation to the data for magnitudes greater than 7.5.

The paucity of data for the largest magnitudes makes it difficult to assess the reliability of the relation we use, which gives moments of  $2 \times 10^{28}$  dyne cm for  $M_s = 7.8$  to  $M_0 =$

**Table 1** Times and locations of the thirty largest earthquakes contributing to the excitation of the Chandler wobble. The magnitude and direction of the pole shift as well as the change in length of day from each earthquake is shown. Depths designated s were assigned a standard value of 33 km. Earth model PEM-A (ref. 16) was used to calculate the pole shifts\*

| No. | Year | Month | Day | Lat.  | Long.  | Depth (km) | Mag | Shift (0.01'') | Dir (°E) | Change in length of day (μs) |
|-----|------|-------|-----|-------|--------|------------|-----|----------------|----------|------------------------------|
| 1   | 1901 | Aug   | 9   | -22.0 | 170.0  | s          | 8.4 | 0.60           | 336.2    | -7.02                        |
| 2   | 1902 | Sep   | 23  | 16.0  | -93.0  | s          | 8.4 | 0.49           | 296.3    | -5.83                        |
| 3   | 1905 | Apr   | 4   | 33.0  | 76.0   | s          | 8.6 | 1.62           | 89.4     | -2.60                        |
| 4   | 1906 | Jan   | 21  | 34.0  | 138.0  | 340        | 8.4 | 0.83           | 101.4    | 1.84                         |
| 5   | 1906 | Jan   | 31  | 1.0   | -81.5  | s          | 8.9 | 1.39           | 218.0    | -160.17                      |
| 6   | 1906 | Aug   | 17  | -33.0 | -72.0  | s          | 8.6 | 2.56           | 108.0    | -16.09                       |
| 7   | 1906 | Sep   | 14  | -7.0  | 149.0  | s          | 8.4 | 0.79           | 239.0    | -0.03                        |
| 8   | 1910 | Jun   | 16  | -19.0 | 169.5  | 100        | 8.6 | 1.78           | 340.7    | -26.53                       |
| 9   | 1911 | Jun   | 15  | 29.0  | 129.0  | 160        | 8.7 | 4.86           | 105.7    | -17.96                       |
| 10  | 1914 | Nov   | 24  | 22.0  | 143.0  | 110        | 8.7 | 3.07           | 160.6    | -43.10                       |
| 11  | 1917 | May   | 1   | -29.0 | -177.0 | 60         | 8.6 | 2.33           | 9.0      | -18.26                       |
| 12  | 1917 | Jun   | 26  | -15.5 | -173.0 | s          | 8.7 | 4.33           | 277.3    | -0.69                        |
| 13  | 1919 | Apr   | 30  | -19.0 | -172.5 | s          | 8.4 | 0.55           | 19.6     | -8.13                        |
| 14  | 1922 | Nov   | 11  | -28.5 | -70.0  | s          | 8.4 | 0.72           | 110.8    | -6.15                        |
| 15  | 1923 | Feb   | 3   | 54.0  | 161.0  | s          | 8.4 | 0.71           | 146.1    | 2.07                         |
| 16  | 1929 | Mar   | 7   | 51.0  | -170.0 | 60         | 8.6 | 2.10           | 171.9    | 6.95                         |
| 17  | 1934 | Jan   | 15  | 26.5  | 86.5   | s          | 8.4 | 0.53           | 95.3     | -3.40                        |
| 18  | 1938 | Nov   | 10  | 55.5  | -158.0 | s          | 8.7 | 3.89           | 189.1    | 17.54                        |
| 19  | 1939 | Dec   | 21  | 0.0   | 123.0  | 150        | 8.6 | 0.84           | 106.7    | -4.36                        |
| 20  | 1940 | May   | 24  | -10.5 | -77.0  | 60         | 8.4 | 0.33           | 73.9     | -8.76                        |
| 21  | 1941 | Jun   | 26  | 12.5  | 92.5   | 60         | 8.7 | 4.30           | 178.4    | -13.57                       |
| 22  | 1941 | Nov   | 25  | 37.5  | -18.5  | s          | 8.4 | 0.53           | 323.6    | -0.27                        |
| 23  | 1942 | Aug   | 24  | -15.0 | -76.0  | 60         | 8.6 | 1.30           | 75.6     | -22.71                       |
| 24  | 1946 | Dec   | 20  | 32.5  | 134.5  | s          | 8.4 | 0.77           | 116.8    | -3.20                        |
| 25  | 1950 | Aug   | 15  | 28.5  | 96.5   | s          | 8.7 | 2.86           | 122.3    | -11.83                       |
| 26  | 1952 | Mar   | 4   | 42.5  | 143.0  | s          | 8.6 | 2.77           | 131.2    | -5.00                        |
| 27  | 1952 | Nov   | 4   | 52.8  | 159.5  | s          | 8.4 | 0.82           | 147.2    | 1.69                         |
| 28  | 1958 | Nov   | 6   | 44.5  | 148.5  | 75         | 8.7 | 4.50           | 130.4    | -2.47                        |
| 29  | 1960 | May   | 22  | -39.5 | -74.5  | s          | 8.6 | 2.56           | 109.2    | -9.80                        |
| 30  | 1964 | Mar   | 28  | 61.1  | -147.6 | 20         | 8.5 | 1.11           | 202.7    | 7.92                         |

\*The indicated pole shifts correspond to a change of the position of the principal axis of the moment of inertia about which the instantaneous rotation axis moves in a circular arc with a 14-month period.

$3.5 \times 10^{30}$  dyne cm for  $M_s = 8.7$ . Nevertheless, these values are grossly consistent with the data, and are all less than the largest moment measured, which was  $\sim 6 \times 10^{30}$  for the 1960 Chilean earthquake<sup>23</sup>. Furthermore, the slope of 2.5 in our equation is consistent with the value of 3 proposed by Kanamori and Anderson<sup>27</sup> for large earthquakes.

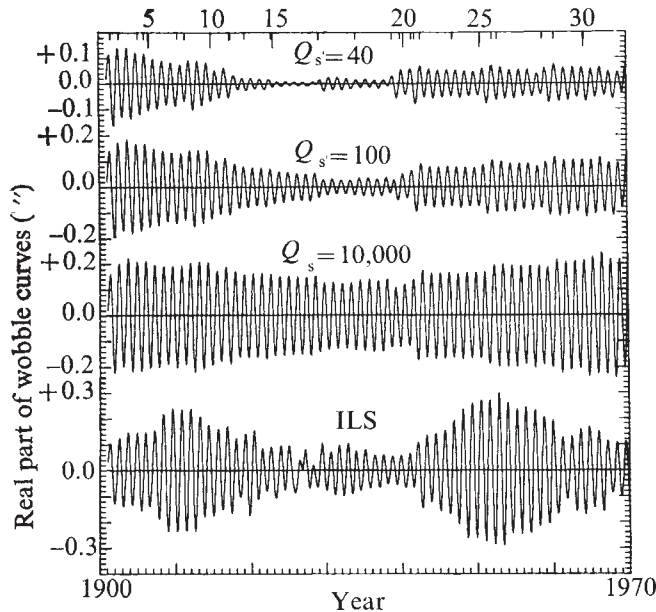


Fig. 2 Simulated polar motion curves resulting from excitation by large earthquakes; the damping for each is determined by  $Q_s$ . ILS is smoothed observed polar motion with annual term removed<sup>30</sup>. Only the component along the Greenwich meridian is shown.

The remaining source parameters were determined from plate tectonic models. Using the data of Solomon *et al.*<sup>28</sup> for the positions of the boundaries and relative motions of the plates, we assign fault plane parameters to earthquakes of three types: normal faulting at mid-ocean ridges (dip =  $45^\circ$ , perpendicular to the ridge axis), transform faulting on fracture zones (dip =  $90^\circ$ ) and thrust faulting at converging plate boundaries (dip =  $20^\circ$ , perpendicular to plate boundary in the direction of the Benioff zone). Such an assignment was straightforward except for large earthquakes in Asia, and those at complex plate boundaries, which were omitted.

The calculated pole shift and direction are given in Table 1 for the 30 largest earthquakes of those considered. Also shown is the change in length of day resulting from the change in the polar moment of inertia of the Earth. For all cases the moments used were derived from the listed magnitudes by use of the relation in Fig. 1. Results for a different magnitude-moment relation can be obtained by appropriate scaling of the magnitude of the pole shift.

Conspicuously absent in Table 1 is the great Sanriku earthquake of March 2, 1933. It occurred near the junction of the Philippine, Pacific and Eurasian plates and it was difficult to assign a particular source mechanism to it. Also, Kanamori<sup>29</sup> has shown that it was not typical of the large shallow earthquakes in the subduction zones; its moment<sup>27</sup> is estimated to be as low as  $4.3 \times 10^{28}$ .

From the calculated pole shifts for 234 earthquakes with magnitudes at least 7.8 between 1901 and 1970 we can compute a synthetic polar wobble curve that would result from this source of excitation alone. For this we also require the starting position  $m(t_0)$  of the pole in January 1901 and the frequency  $\omega_0$  and damping,  $Q$  of the Chandler wobble.

The synthetic wobble  $m(t)$  is computed from

$$m(t) = m(t_0) \exp[i\omega(t-t_0)] + \sum_k s_k H(t-t_k) \left\{ 1 - \left( 1 + \frac{\omega_0}{\Omega} \right) \exp[i\omega(t-t_k)] \right\}$$

where  $\omega$  is the complex circular frequency of the Chandler wobble:  $\omega = \omega_0(1+i/2Q)$ ,  $s_k$  are the complex pole shifts caused by earthquakes at times  $t_k$ ,  $\Omega$  is the frequency of the diurnal rotation and  $H$  is the unit step function<sup>12</sup>. The complex function  $m(t)$  is referred to the coordinate system in which the real axis corresponds to the Greenwich meridian and the imaginary axis points towards  $90^\circ\text{E}$ .

Figure 2 shows the real part ( $m_x$ ) of three such synthetic wobble curves obtained using  $\omega_0 = 5.287$  radians  $\text{yr}^{-1}$  after Wilson<sup>30</sup> and three different values of  $Q$ . Shown for comparison is a smoothed version of the ILS polar motion data, with the annual term removed, also from Wilson<sup>30</sup>. The initial pole location is the same for all curves, and each curve has been bandpass filtered from 0.3 to 0.98 cycles  $\text{yr}^{-1}$  to facilitate comparison. The ticks on the top of the figure mark the times of the earthquakes in Table 1.

The general trend of the amplitude of the wobble can be seen in the ILS data. The observed amplitude was large in 1910, decreased fairly uniformly from 1910 to 1920 and remained small until 1940 when it began to increase again to reach the maximum value by 1954. Since then there has been an irregular decline in amplitude. The synthetic wobble time series, particularly for  $Q = 100$ , reproduce the principal features of these amplitude changes. The decrease in the synthetic wobble is caused by the cumulative effect of several large earthquakes from 1914 to 1929. This occurs even for the case with  $Q = 10,000$  and it is thus apparent that earthquakes can reduce the amplitude of the wobble and a rapid decay of amplitude need

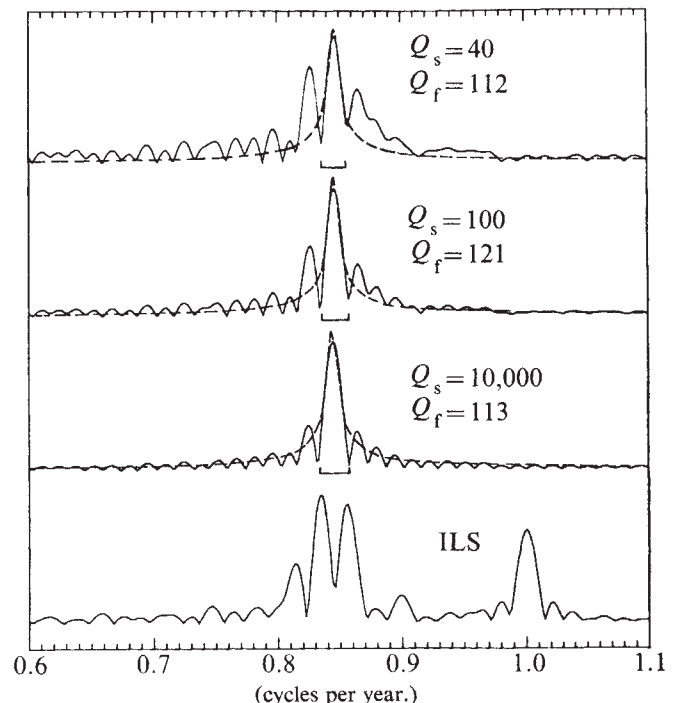


Fig. 3 Power spectra (—) of the simulated and observed (ILS) polar motion shown in Fig. 2. Resonance curves were fitted to the central peaks to determine  $Q_r$  (---).

not be attributed to intrinsic dissipation. Similarly, the increase in the synthetic wobble amplitude arises from large earthquakes from 1938 through 1950 which gradually re-excite the wobble. We should note that the decline in amplitude observed since



1954 is not reproduced in the synthetic wobble curves; this may be an effect of other sources of excitation or accumulation of the phase shift due to a difference between the actual  $\omega_0$  and the value of Wilson<sup>30</sup>.

The power spectra of the three synthetic time functions  $m(t)$  are compared in Fig. 3 with the power spectra of the ILS data. Although it is clear that we were not able to reproduce exactly the observed spectra, the synthetic curves for  $Q_s = 40$  and  $Q_s = 100$ , each having three well developed maxima, exhibit a similar order of complexity. Also shown is a least squares fit of a resonance curve to the central peak of the synthetic series, which yields an estimate  $Q_t$  that is nearly independent of the intrinsic  $Q_s$  of the model. This illustrates the fact that a damping of an episodically excited harmonic oscillator cannot be estimated from its spectrum, unless specific assumptions about the nature of the excitation are made<sup>31</sup>. It is clear that reliable estimates of both the frequency and damping of the wobble may depend on the detailed nature of the excitation, especially if it is dominated by a relatively few large impulsive events. Thus an accurate evaluation of the excitation from earthquakes (or other sources) may permit determination of the effective elastic and anelastic properties of the earth at the Chandler wobble period sufficiently precise for comparison with those inferred from seismic observations.

## Discussion

The fact that synthetic wobble calculations reproduce the principal variations of amplitude of the observed Chandler wobble substantiates the correlation between global seismicity and changes in the amplitude of the wobble noted by Myerson<sup>8</sup>. Since a given earthquake can either increase or decrease the amplitude of the wobble, the fact that the cumulative effect of several earthquakes is in accord with observations strongly suggests that the correlation is indeed real, and that earthquakes make a noticeable contribution to the excitation of the wobble. Comparison of amplitudes of the synthetic and observed wobble suggests that the earthquake series, with the specific moments assigned through our magnitude-moment relationship could account for  $\sim \frac{1}{2}$  of the excitation; the remainder could come from other sources. For example, Wilson<sup>30</sup> has stated that the atmosphere can account for  $\sim \frac{1}{2}$  of the observed wobble, and possibly more. If this is true, then the relative contributions to the excitation of the wobble by the atmosphere and earthquakes could be of comparable magnitude.

The magnitude of the seismic excitation exhibited by the synthetic wobble curves depends on the moments assigned to the individual earthquakes. These are quite uncertain, as can be seen by the scatter of points around the lines in Fig. 1. Furthermore, it must be noted that the moments calculated here for some of the earthquakes before 1960 are larger than have been previously reported for the same events (see Table 1 in ref. 27). The moments of earlier earthquakes may have been underestimated because of the lack of adequate long period instrumentation that would allow measurements of amplitudes of surface waves and free oscillations at periods of at least several hundred seconds, so that their wavelengths would be significantly greater than the source dimensions.

While seismic moments are usually estimated from the radiation of the seismic waves associated with the main shock, excitation of the wobble would be affected by the displacements that may take place over a relatively long period of time (several weeks) before and after an event. For example, Kanamori and Cipar<sup>23</sup> identified an 'aseismic' event several minutes before the main Chilean shock (1960); the moment of this event could be equal to, or perhaps even exceed, that of the main shock<sup>32</sup>. Dziewonski and Gilbert<sup>18</sup> reported release of isotropic stress preceding two deep earthquakes; the spectra indicated the aseismic character of these events. Such 'silent earthquakes', whether or not they are followed by major seismic events, may be more common than previously believed. In fact, such a conclusion is suggested by the work of Davies

and Brune<sup>33</sup>, who compared the cumulative seismic moment release with the average plate tectonic displacement rates at converging plate boundaries. Although they found a good correspondence between cumulative moment release and plate-tectonic slip rates, this only held for shallow earthquakes with an assumed depth of faulting  $< 60$  km. They noted that differential motion at greater depths seems to be accomplished by aseismic deformation. Such deformation may well be associated with large earthquakes and would increase the total shift of the pole over that calculated from the observed amplitudes of seismic waves. In fact the static moments associated with great earthquakes, or aseismic events, are sufficiently uncertain that one cannot rule out the possibility that these account for most of the excitation of the wobble.

This conclusion is not in agreement with that earlier reached by Dahlen<sup>8</sup>. At that time no earthquake had been assigned a moment greater than  $10^{30}$  dyne cm; which led him to restrict the maximum allowed moment to that value. Subsequent redeterminations of the seismic moment of the great Chilean earthquake<sup>23,32</sup> relaxes this restriction and makes plausible the implications of the moment-magnitude relationship used in this report. This, in turn, leads to the calculated excitation comparable to that observed. We note, however, that the 70-yr average of the polar shifts from earthquakes listed in Table 1 is only  $0.008'' \text{ yr}^{-1}$ ; approximately equal to that computed by Dahlen<sup>8</sup> for the 1964 Alaskan earthquake.

The results of this study indicate that the cumulative effects of major earthquakes can account for at least a large part of the excitation of the Chandler wobble, and in particular for the long term variations of the amplitude of the wobble. This, together with recent estimates of the atmospheric excitation of the wobble<sup>30</sup>, indicates that these two mechanisms may well account for the entire excitation. If this is verified by more detailed analysis, it should permit more accurate determination of the period and damping of the wobble, and of the effective elastic properties of the Earth at very long periods.

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# Possible evidence for penetration of interstellar dust into the Solar System

J. L. Bertaux & J. E. Blamont

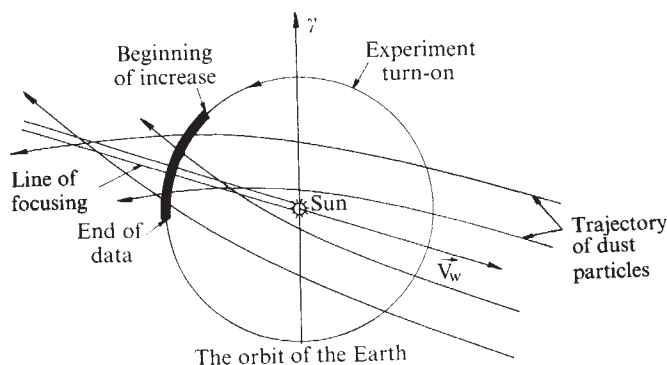
CNRS, Service d'Aéronomie, BP No 3-91370 Verrières Le Buisson, France

*Because of the motion of the Sun through the surrounding interstellar medium, interstellar dust particles may penetrate deep into the Solar System. In this paper, we investigate the conditions for this penetration and find that the distribution of such particles should peak along the line of gravitational focusing. Two possible consequences are compared with experimental results: the rate at which particles should strike a micrometeoroid detector and the distribution of scattered solar light. We find that the number density of particles penetrating the solar system, derived from this comparison, is much smaller than predicted by a crude model of interstellar matter.*

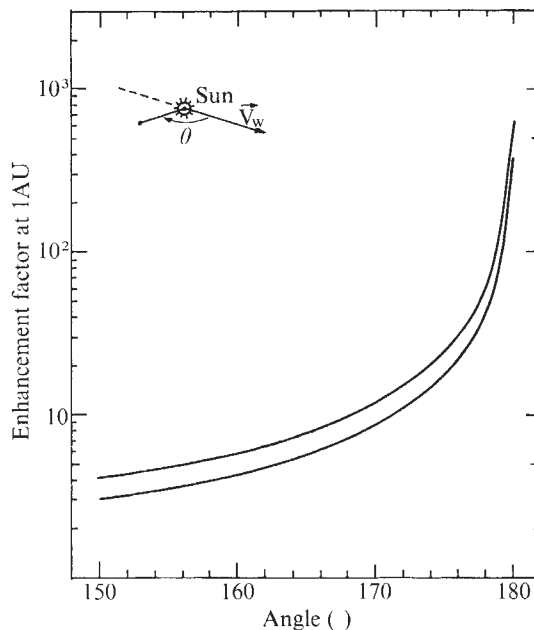
THE velocity of the Solar System relative to local interstellar matter has recently been determined by observation on the interplanetary emissions of hydrogen at 1,216 Å (refs 1 and 2) and of helium at 584 Å (ref. 3). These show that the Solar System is moving through the interstellar gas with a velocity<sup>4</sup> of  $\sim 20 \text{ km s}^{-1}$  in the direction<sup>3</sup>  $\alpha = 252^\circ$ ,  $\delta = -15^\circ$  (equatorial coordinates), different from the apex direction by  $\sim 50^\circ$ , lying in the galactic plane and near  $(7 \pm 3^\circ)$  the ecliptic plane (Fig. 1).

The measured density of H (0.1 atoms  $\text{cm}^{-3}$ ) and He (0.01 atoms  $\text{cm}^{-3}$ ) and the high temperature of H found by Bertaux *et al.*<sup>4</sup> with the Soviet probe Mars-7  $((6-13) \times 10^3 \text{ K})$  indicate that the Solar System is not now moving through an interstellar cloud, but through the so-called intercloud medium. These results indicate that the lack of hydrogen found around the Gum Nebula by the Copernicus measurements<sup>5</sup> does not hold for the immediate vicinity of the Solar System.

Matter penetrating into the heliosphere is subjected to two



**Fig. 1** The trajectories of interstellar dust particles are focused in the Solar System along a line opposite to  $V_w$ , the velocity vector of the Solar System relative to the interstellar matter. As  $V_w$  is near the ecliptic plane ( $7 \pm 3^\circ$ ), the Earth intersects it around December 1, a time of an increased density interstellar dust, which, presumably, has been detected by the MTS micrometeoroids instrument.



**Fig. 2** The enhancement factor of dust particle density in the Solar System caused by gravitational focusing, is computed at 1 AU as a function of angle  $\theta$  with  $V_w$ , for two values of  $p$  (the ratio of radiation pressure to gravitation)  $p = 0$  (upper curve) and  $p = 0.5$  (lower curve).

opposing inverse square forces, gravity and radiation pressure. The latter dominates for hydrogen atoms, which are deviated by and from the Sun along divergent hyperbolae, and the former dominates for all heavier atoms including helium atoms: gravitational focusing creates a zone of increased density in the downwind region of the Solar System, along the line  $-V_w$  (Fig. 1). Both depletion of hydrogen and enhancement of helium have been observed in the Sun's wake.

Since interstellar matter contains dust as well as gas, gravitational focusing will cause an increase in the number density of dust grains along the direction  $-V_w$  ( $\alpha = 72^\circ$ ,  $\delta = +15^\circ$ ). Such an increase should be detectable when the Earth's orbit takes it across this region of space, that is, from November to January.

## Distribution of dust grains

We have used a computer program, which we had developed originally to determine the density distribution of interstellar hydrogen and helium in the interplanetary medium, to compute the density distribution of dust grains in the Solar System as a function of the distance to the Sun and the angle  $\theta$ , measured from the direction  $V_w$  of the incident beam (Fig. 2, inset). In this coordinate system, gravitational focusing occurs for  $\theta \simeq 180^\circ$ . Figure 2 shows the density enhancement factor,  $E$ , at 1 AU as a function of  $\theta$  from  $150^\circ$  to  $180^\circ$ . This factor is defined as the position-dependent number density of particles when solar forces are taken into account, relative to the density of particles in the nearby interstellar medium. The upper curve



**Table 1** Parameters of the grains of interstellar dust used in this paper

|                                                  | Graphite covered with ice | Graphite              | Silicate              |
|--------------------------------------------------|---------------------------|-----------------------|-----------------------|
| Relative number                                  | 1                         | $10^2$                | $10^3$                |
| Radius ( $\mu\text{m}$ )                         | 0.5                       | 0.02                  | 0.02                  |
| Density of particles ( $\text{g cm}^{-3}$ )      | 2                         | 2                     | 2.5                   |
| Mass of particle (g)                             | $1.04 \times 10^{-12}$    | $6.7 \times 10^{-17}$ | $8.4 \times 10^{-17}$ |
| Mass distribution ( $\text{g cm}^{-3}$ )         | $1.6 \times 10^{-27}$     | $10^{-28}$            | $1.3 \times 10^{-28}$ |
| Mass distribution (%)                            | 92                        | 0.6                   | 7                     |
| Number density of particles ( $\text{cm}^{-3}$ ) | $1.5 \times 10^{-15}$     | $1.5 \times 10^{-13}$ | $1.5 \times 10^{-12}$ |
| $p$ = radiation pressure/gravity                 | $> 1$                     | $> 1$                 | $< 1$                 |

applies for negligible radiation pressure, the lower for a radiation pressure equal to half the gravitational force. These curves were calculated for dust grains with an isotropic velocity dispersion of  $0.1 \text{ km s}^{-1}$  added to their bulk velocity of  $20 \text{ km s}^{-1}$ ; a dispersion of  $1 \text{ km s}^{-1}$  gives almost the same result.

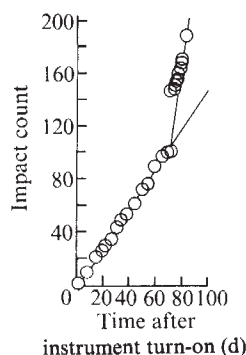
### Our model of the interstellar dust

To evaluate quantitatively the possible effects of such a density enhancement, we adopted a crude model of the interstellar dust. The mass ratio of dust to gas was taken as  $10^{-2}$  and the dust was supposed to contain three types of particles: 1 graphite particle covered with ice (radius  $0.5 \mu\text{m}$ ) to  $10^2$  graphite particles (radius  $0.02 \mu\text{m}$ ) to  $10^3$  silicate particles (radius  $0.02 \mu\text{m}$ ). The resulting mass and number distribution of these particles in the interstellar medium in the vicinity of the Sun are shown in Table 1. Since the hydrogen number density is  $0.1 \text{ atoms cm}^{-3}$ , or  $1.7 \times 10^{-25} \text{ g cm}^{-3}$ , the total dust density is  $\approx 1.7 \times 10^{-27} \text{ g cm}^{-3}$  on our model.

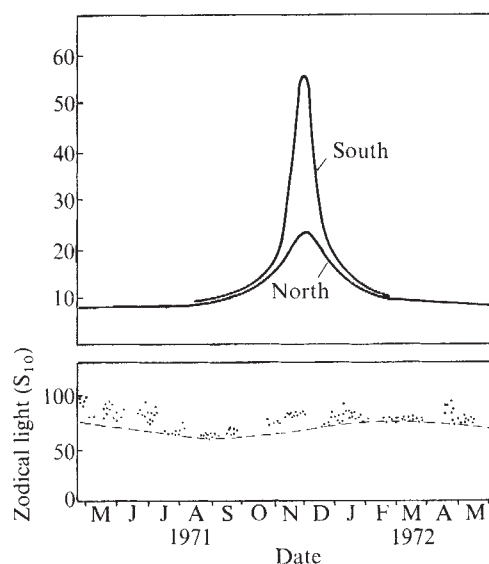
The ratio of radiation pressure to gravity—the last figure in Table 1—depends on the size and type of particle and is a crucial parameter in our study. For the first two types of particles, this ratio is greater than one<sup>6</sup>; they do not penetrate deeply inside the Solar System and are not subjected to gravitational focusing. For non-metallic and non-absorbing material, like silicate particles, this ratio is, however, less than one. This is also true for quartz and obsidian particles<sup>7</sup>. Radiation pressure acts as a differentiation process for interstellar matter penetrating the Solar System. Only particles with  $p < 1$  will be focused along the downwind axis.

### Consequences: (1) micrometeoroids

We invoke this enhancement to explain the observations reported by Alvarez<sup>8</sup> using two micrometeoroid detectors of the MOS capacitor type in terrestrial orbit on board the MTS satellite. The number of impacts as a function of time is shown in Fig. 3. The count rate (slope of the curve) presents a sharp



**Fig. 3** The cumulative count for impacts detected by the MTS micrometeoroid instrument plotted as a function of time after the second instrument turn-on, which occurred 2 yr. after launch. An initial penetration rate of  $4.3 \times 10^{-4} \text{ m}^{-2} \text{ s}^{-1}$  (slope of lower line) becomes  $2.0 \times 10^{-3} \text{ m}^{-2} \text{ s}^{-1}$ .



**Fig. 4** D2-A observations of zodiacal light in the direction of North ecliptic pole (lower graph) show a bump in November and December, compared with the dashed line showing the expected value. According to the model computation shown in the upper graph, this bump might be produced by the penetration of interstellar dust into the Solar System, concentrated along the line of gravitational focusing, characterised by  $n_d \sigma = 2 \times 10^{-23} \text{ cm}^{-1}$ .

increase (by a factor of 5) around the beginning of November which corresponds to an ecliptic longitude  $\lambda = 43^\circ$  for the Earth (Fig. 1). The increase persists up to at least  $\lambda = 93^\circ$ —just what is expected from the location of the line  $-V_w$  (Fig. 1).

From the maximum count rate  $P_{\text{max}} = 2 \times 10^{-3} \text{ m}^{-2} \text{ s}^{-1}$ , and our simple model of gravitational focusing, we may infer the fraction of dust in the near interstellar medium for which  $p < 1$ . Particles with a velocity  $V_w$  of  $20 \text{ km s}^{-1}$  at infinity have a velocity at 1 AU  $V_1 \approx 46 \text{ km s}^{-1}$  (from conservation of energy). For this velocity one of the two MTS detectors is sensitive to particles with mass  $m > 3 \times 10^{-17} \text{ g}$ , whereas the other is sensitive to  $m > 3 \times 10^{-16} \text{ g}$ . The second detector also showed an enhancement at the same time, but somewhat less pronounced. Thus we estimate that the enhancement detected by the MTS instruments comes mainly from particles of interstellar origin with a mass  $m \approx 2 \times 10^{-16} \text{ g}$ ; this corresponds to a particle radius of  $\sim 0.03 \mu\text{m}$ . If  $n_d$  is the number of particles in the incident beam (per  $\text{cm}^3$ ) the flux,  $F$ , at 1 AU in the region of enhancement is

$$F = n_d E V_1$$

since  $E = 10$ ,  $V_1 = 46 \text{ km s}^{-1}$ ,  $F = 2 \times 10^{-7} \text{ cm}^{-2} \text{ s}^{-1}$  we find

$$n_d \approx 4.3 \times 10^{-15} \text{ cm}^{-3}$$

and a dust density of  $\sim 10^{-30} \text{ g cm}^{-3}$

### Possible explanations

These numbers are very much lower than our crude model predicts, even if only silicate particles are considered. To explain this discrepancy, it may be that:

- (1) MTS detectors are sensitive to only a small fraction of the interstellar dust particles which penetrate the Solar System;
- (2) radiation pressure (or some other process) eliminates most of the particles that could have been detected by MTS; or
- (3) the interstellar dust-gas ratio around the Sun is smaller than the galactic mean, which could be connected to the embedding of the Solar System in the 'intercloud' type of interstellar matter.

### Consequences: (2) zodiacal light

We have also tried to compute the contribution to zodiacal light of solar light scattered by the interstellar particles inside the Solar System. Only spherical silicate particles were considered, on which the effect of radiation pressure was assumed to be negligible. For any point in the Solar System ( $r, \theta$ ) the emissivity  $\epsilon(r, \theta)$  is

$$\epsilon(r, \theta) = n_d E(r, \theta) \sigma F_s \left( \frac{r_0}{r} \right)^2 \quad (1)$$

where  $\sigma$  is the scattering cross section and  $F_s$  the solar flux at  $r_0 = 1$  AU. If the scattering is assumed to be isotropic, the intensity is

$$I = \frac{1}{4\pi} \int \epsilon(l) dl \quad (2)$$

If, therefore, all particles have the same  $\sigma$ , the zodiacal intensity caused by interstellar dust is proportional to  $n_d \sigma$ .

The intensity at 1 AU was computed in the direction of North and South ecliptic poles, as a function of Earth's position (or time of the year). It is shown in Fig. 4, together with observations<sup>9</sup> of zodiacal light collected by a photometer in the French satellite D2-A in the direction of North ecliptic pole during the year 1971-72. The solar flux in equation (1) was taken as  $F_s = 6.2 \times 10^{13}$  photons  $\text{cm}^{-2} \text{s}^{-1} \text{\AA}^{-1}$ , at the effective wavelength of the D2-A photometer (6,563 Å). The intensity is measured in  $S_{10}$  units (equivalent brightness of one tenth magnitude star per square degree;  $1 S_{10} = 360 \text{ photons cm}^{-2} \text{s}^{-1} \text{\AA}^{-1} \text{sr}^{-1}$ ).

The dashed line traced through the D2-A points represents the expected variation of zodiacal light, since the Earth's orbital plane does not coincide exactly with the symmetry plane of the zodiacal cloud<sup>9</sup>. The data points in November and December are  $\sim 15 S_{10}$  above this line. This 'bump' agrees with the maximum computed for light scattered by interstellar material. For the model computation, the value of  $n_d \sigma$  was adjusted to  $2 \times 10^{-23} \text{ cm}^{-1}$ , which also gives for the bumps computed for the North pole, a value of  $15 S_{10}$ . The observations of zodiacal light in this direction therefore provide information about the product  $n_d \sigma$ . If the bump in the measurements is genuine, we have  $n_d \sigma \approx 2 \times 10^{-23} \text{ cm}^{-1}$ . If the bump is accidental, this value for  $n_d$  becomes an upper limit.

Since the line of gravitational focusing lies  $\sim 7^\circ \text{S}$  of the ecliptic plane, a stronger effect is expected in the direction of the South ecliptic pole, with an increase of  $\sim 50 S_{10}$  at the time of maximum (Fig. 4). Unfortunately, South ecliptic pole

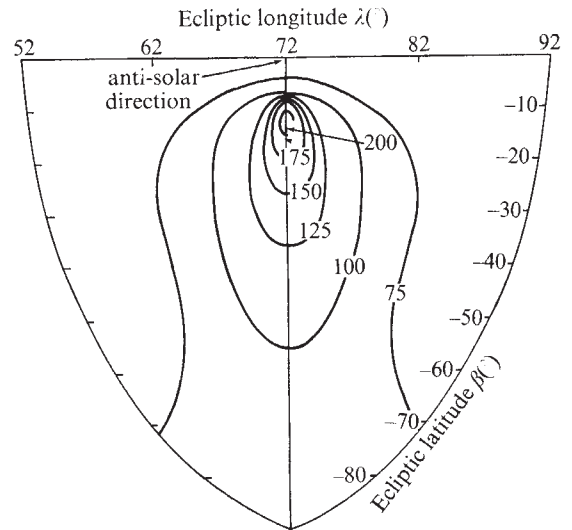


Fig. 5 Isophotes of the 'trail of light' (which would be superimposed on the zodiacal light pattern, when observed from Earth on December 1, south of the anti-solar direction) produced by the gravitational focusing of interstellar dust particles characterised by  $n_d = 2 \times 10^{-23} \text{ cm}^{-1}$ . Intensity levels are in units of  $S_{10}$  (see text).

observations are hampered by the presence of the Magellanic clouds. For an observatory attached to the Earth, the strongest intensity of light by interstellar dust should be observed not far from the line of gravitational focusing. This would happen around December 1, when the Earth is at an ecliptic longitude  $\gamma = 72^\circ$ , in the general anti-solar direction. For these conditions, the distribution of intensity scattered by interstellar material was computed with the use of equations (1) and (2) taking  $n_d \sigma = 2 \times 10^{-23} \text{ cm}^{-1}$ .

The maximum intensity appears at the ecliptic longitude of  $\gamma = 72^\circ$ , slightly south of the ecliptic latitude  $\beta = -7^\circ$  (which is the direction of gravitational focusing) because of the effect of perspective. As the longitude scale of Fig. 5 is five times larger than the latitude scale, the 'true' shape of such a conspicuous feature would be much narrower, like a pencil of light.

The maximum brightness of this feature, which would be superimposed on the usual zodiacal light pattern, is  $\sim 200 S_{10}$ , to be compared with a mean gegenschein brightness of  $180 S_{10}$ . Unfortunately, gegenschein observations close to December 1 are scarce, because of a strong galactic background in the anti-solar direction at this time. From a long series of gegenschein observations obtained at Tenerife, however, Dumont (personal communication) estimated that if such a feature were brighter than  $50 S_{10}$ , it would have been observed. Therefore, one can derive another upper limit for  $n_d \sigma$ ,  $5 \times 10^{-24} \text{ cm}^{-1}$ , which is more stringent than the one derived from the D2-A observations, and will serve as a basis for discussion in the following (if the November-December bump of D2-A observations was revealed to be connected with interstellar dust, one would have to explain this discrepancy with gegenschein observations).

Table 2 Parameters for the scattering of zodiacal light

| Particle radius, $a(10^{-8} \text{ m})$ | $x = \frac{2\pi a}{\gamma}$ | Scattering cross section $\rho \text{ (cm}^{-2}\text{)}$ | Particle mass (g)     | Optical upper limit on $n_d$ | $n_d m \text{ (g cm}^{-3}\text{)}$ |
|-----------------------------------------|-----------------------------|----------------------------------------------------------|-----------------------|------------------------------|------------------------------------|
| 1.75                                    | 0.2                         | $4.3 \times 10^{-15}$                                    | $5.6 \times 10^{-17}$ | $1.2 \times 10^{-9}$         | $6.7 \times 10^{-26}$              |
| 3.5                                     | 0.4                         | $2.7 \times 10^{-13}$                                    | $4.5 \times 10^{-16}$ | $1.9 \times 10^{-11}$        | $8.5 \times 10^{-27}$              |
| 5.2                                     | 0.6                         | $3.1 \times 10^{-12}$                                    | $1.5 \times 10^{-15}$ | $1.6 \times 10^{-12}$        | $2.4 \times 10^{-27}$              |
| 7                                       | 0.8                         | $1.7 \times 10^{-11}$                                    | $3.6 \times 10^{-15}$ | $2.8 \times 10^{-13}$        | $1.0 \times 10^{-27}$              |
| 8.7                                     | 1.0                         | $6.2 \times 10^{-11}$                                    | $7 \times 10^{-15}$   | $8.1 \times 10^{-14}$        | $5.7 \times 10^{-28}$              |



## Discussion

As the scattering cross section  $\sigma$  is a strong function of the radius of particle  $a$ , the upper limit on  $n_a\sigma$  gives an upper limit on  $n_a$  which is a function of  $a$ , or  $x = 2\pi a/\lambda$  (Table 2). The optical characteristics of spherical silicate particles are taken from Wickramasinghe<sup>10</sup>:  $n = 1.55$ ,  $k = 0$ , at  $\lambda = 6,560 \text{ \AA}$ .

Even with a particle radius approaching  $0.1 \text{ \mu m}$  (last line of Table 2), the optical upper limit on  $n_a$ ,  $8.1 \times 10^{-14} \text{ cm}^{-3}$ , is still  $\sim 20$  times larger than that derived from the micrometeoroid rate as measured by MTS detectors. Optical observations do not therefore contradict, up to now, MTS observations.

Table 2 indicates that the penetration into the Solar System of interstellar silicate particles with an assumed number density of  $1.5 \times 10^{-12} \text{ cm}^{-3}$  (as in our crude model) would have been observed optically only if these particles had a radius larger than  $0.05 \text{ \mu m}$  (which goes against our assumptions). This crude model is therefore consistent with the absence of a

conspicuous 'trail of light' along the line of gravitational focusing. The best way to detect interstellar dust particles in the Solar System would probably be to launch a space probe equipped with micrometeoroid detectors on a trajectory intersecting the line of gravitational focusing.

We are indebted to Dr Alvarez for early communication of his results and to Dr Dumont, for discussions of his observations of zodiacal light.

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# Neutron diffraction studies of retinal rod outer segment membranes

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*Neutron diffraction measurements on isolated retinal rod outer segments show that most of the visual pigment protein, rhodopsin, is embedded in the hydrophobic core of the disk membrane. A very slight outward shift of protein at the cytoplasmic side of the membrane is associated with pigment bleaching.*

STRUCTURAL analysis of retinal rod outer segment (ROS) membranes by X-ray diffraction has indicated that rhodopsin, which constitutes almost all the membrane protein, is partially or wholly immersed in the hydrophobic layer of the membrane, but details of its distribution are not unequivocally determined<sup>1-7</sup>. Differences in lamellar diffraction intensities between dark-adapted and bleached membranes<sup>4,5</sup> are interpreted as due to a small increase in electron density near the cytoplasmic (interdisk) side of the membrane. Movement of rhodopsin into the membrane on bleaching was deduced from measurements of diffraction in the plane of the membrane<sup>8</sup>, but these measurements have been questioned<sup>7</sup>. Information from X-ray diffraction studies is limited by the imperfect stacking of the membranes, the low contrasts of electron densities among the components of the lipid-protein-water structure, and by the difficulty of placing electron density profiles on an absolute scale. Consequently, features in the electron density profile and changes on bleaching cannot be attributed with certainty to particular membrane components.

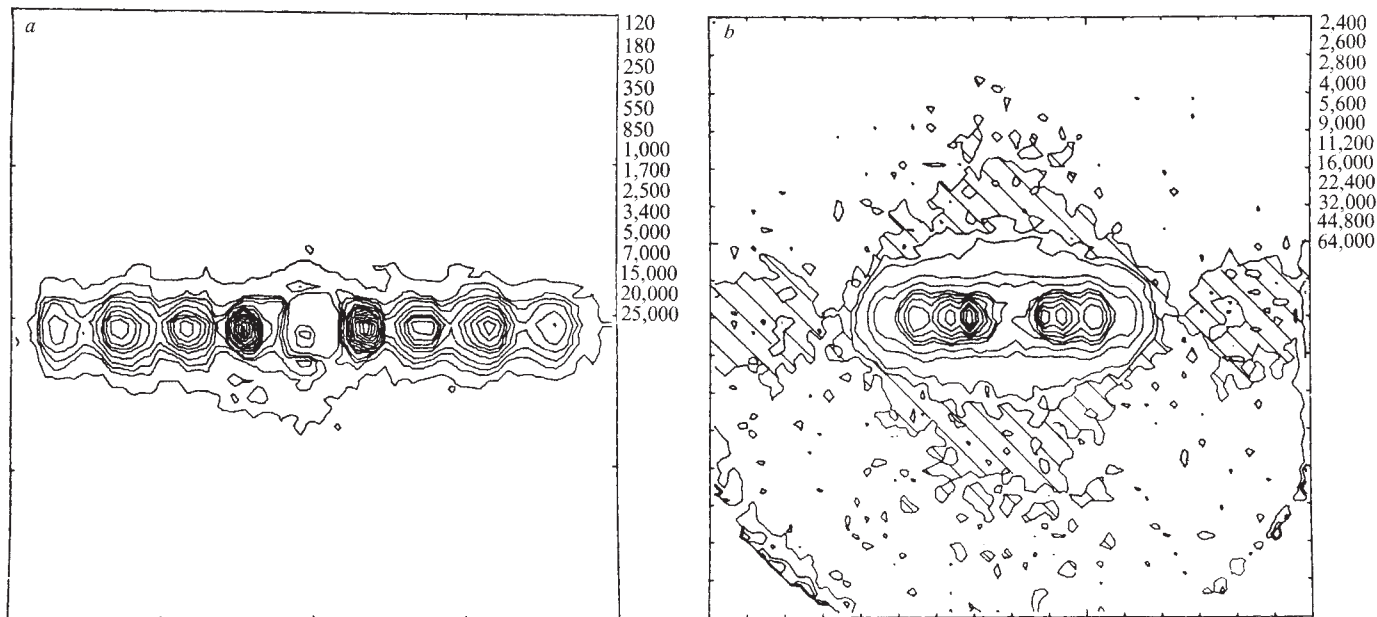
With the development of the high flux neutron beam facilities of the Institut Laue-Langevin, Grenoble, and the low angle scattering instrument D-11 (ref. 9), it has become possible to record neutron diffraction from small samples in times comparable with those of X-ray diffraction. A major advantage of neutron scattering studies is that the neutron

scattering densities of biological membrane components are between those of  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$ . Therefore, the range of contrast between the cytoplasm and the membrane can be varied from positive to negative by varying the  $\text{H}_2\text{O}/\text{D}_2\text{O}$  content of the Ringer's solution. The linear relationship between structure factors and  $\text{D}_2\text{O}$  percentage establishes the variation of phase of each order with contrast, using diffraction intensities from a single sample in at least three different  $\text{H}_2\text{O}/\text{D}_2\text{O}$  mixtures<sup>10,11</sup>. Furthermore, contrast variation data together with the existence of a clearly defined water layer provide an absolute scale of neutron scattering density. Using these neutron diffraction methods<sup>12</sup>, we have obtained new, quantitative information on the distribution of proteins, lipids and water in the ROS disk membrane, and on structural changes associated with bleaching of the visual pigment.

## Lamellar organisation in dark-adapted ROS membranes

Seven orders of lamellar neutron diffraction were measured in  $\text{H}_2\text{O}$  and 20%, 40% and 100%  $\text{D}_2\text{O}$  Ringer solutions from isolated frog rods prepared and oriented using a magnetic field, as described previously<sup>7</sup>. Figure 1 shows contour maps of neutron diffraction patterns: a  $\text{D}_2\text{O}$  pattern showing four lamellar diffraction orders (a); and an  $\text{H}_2\text{O}$  pattern with seven lamellar diffraction orders and diffuse scatter at right angles to the lamellar diffraction visible (b). Orders 1-4 are very intense in  $\text{H}_2\text{O}$ , 40% and 100%  $\text{D}_2\text{O}$  and are about 10 times larger than the higher orders. In 20%  $\text{D}_2\text{O}$ , which has a scattering density close to the average scattering density of the membrane, the pattern is very weak and orders 6 and 7 are prominent.

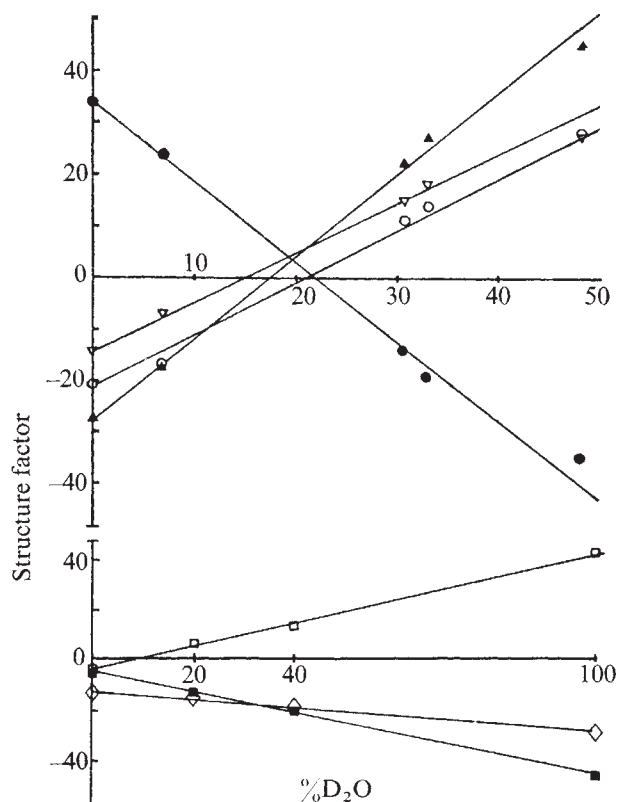
For phase assignment, the large range of contrast available



**Fig. 1** Neutron diffraction patterns from freshly isolated frog ROS oriented using a magnetic field of 17 kgauss are shown in these contour maps of data obtained with the two-dimensional ( $64 \times 64$  cm) detector of the low angle scattering instrument D-11 at the Institut Laue-Langevin<sup>9</sup>. The contour lines connect points of equal diffraction intensity, and the numbers beside the maps refer to the number of counts in successive contour levels. The distribution of neutron wavelengths was triangular, with  $\Delta\lambda/\lambda$  (FWHM) = 8%. Rods from 10–12 retinæ were suspended in Ringer's solution, in a standard quartz spectrophotometer cell of 2 mm path length, and allowed to sediment in a horizontal magnetic field, forming an oriented pellet 1 mm thick. The long axes of the rods aligned along the field direction, and therefore the disk membranes were perpendicular to the field. The lamellar spacing was 295 Å, and the unit cell is centrosymmetric. *a*, Diffraction pattern in  $D_2O$  Ringer with a neutron wavelength of 9 Å. Four orders of lamellar diffraction are visible on each side of the central beam stop. The counting time was 10 min. The mosaic spread is less than  $10^\circ$  (FWHM). *b*, Diffraction pattern in  $H_2O$  Ringer, with a neutron wavelength of 4.4 Å. Lamellar orders up to 7 and broad in-plane diffraction are visible. Order 1, is partly masked by the beam stop. The counting time was 1 h. Shading has been added to the region bounded by the lowest intensity contours to make order 7 and in-plane diffraction clearer.

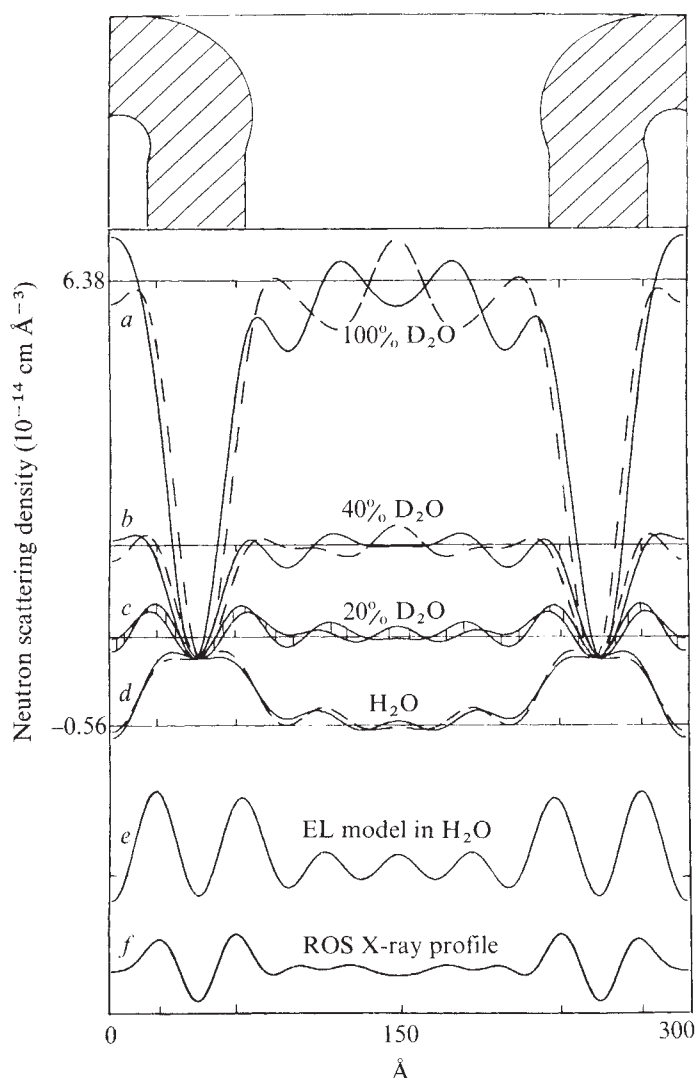
enabled simple deductions: (1) In  $D_2O$ , every component of the membrane appears with negative contrast and orders 1–4 are prominent. Of all the possible phase factor combinations for those four orders, only one ( $-++$ ) gave a reasonable position in the unit cell, consistent with X-ray

and electron microscopy results, for a negatively contrasting membrane. (2) In 20%  $D_2O$ , the relative neutron scattering densities of protein, phospholipid headgroups, hydrocarbon chains and Ringer's solution are close to their relative electron densities. Therefore, the diffraction intensities and the phases should be the same for neutron scattering in 20%  $D_2O$  and X-ray scattering. In fact, the neutron diffraction pattern in 20%  $D_2O$  is very similar to the X-ray pattern. We conclude that the phases are also the same. The X-ray phases have been determined by osmotic swelling<sup>3-5</sup>. These phases for the neutron pattern in 20%  $D_2O$  and the linearity of structure factor with  $D_2O$  percentage (Fig. 2) give the phase of each order at all contrasts. The above two procedures gave consistent phase assignments for all orders except the very weak fifth order. An experiment with osmotic shrinking in  $D_2O$  indicated the presence of a zero in the



**Fig. 2** Contrast variation. Structure factors 1–7 are plotted against the  $D_2O$  percentage of the Ringer's solution to establish phase changes between  $H_2O$  and  $D_2O$ . The data for orders 1–4 (●, 1; ○, 2; ▲, 3; ▼, 4) were measured with the same sample, to keep a fixed intensity scale. To change the contrast at the end of a measurement, a known amount of the supernatant solution was removed from the sample cell without losing or bleaching any rods, and replaced by a different solution. The rods were then resuspended in the new mixture, reoriented in the magnetic field and allowed to resediment. This process was completed in a few minutes. Orders 5–7 (□, 5; ■, 6; ◇, 7) were scaled from longer measurements on other samples by normalising to order 3. Intensities ( $I_h$ ) were obtained by integrating the lamellar diffraction peaks over their arcs of mosaic spread, and multiplying by a Lorentz factor of  $h$ , giving structure factors  $F_h = \sqrt{hI_h}$ . To obtain the intensities of the higher orders, the peak shapes along the lamellar axis were approximated by Gaussians of half-width calculated from the beam size, wavelength broadening and natural widths due to the lattice disorder (natural widths obtained from X-ray data). In 20%  $D_2O$ , both the intensity, which was very weak, and the phase of order 5, were uncertain.

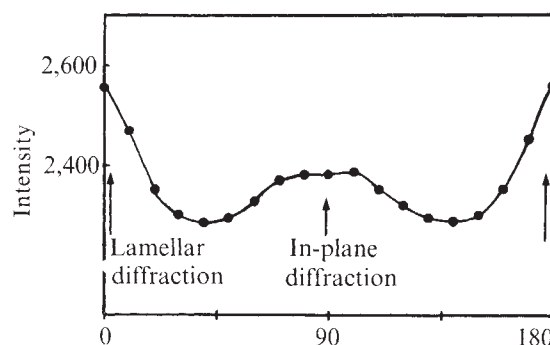




**Fig. 3** *a-d*, Neutron scattering density profiles of ROS computed with seven orders of diffraction, in 100%  $D_2O$ , 40%  $D_2O$ , 20%  $D_2O$  and  $H_2O$  Ringer's solutions. Structure factors used are those shown in Fig. 2. *e*, Alternative phasing of order 5. Order 5 is taken as zero in the 20%  $D_2O$  profile (*c*), with upper and lower limiting profiles shown. Absolute density scaling: The structure factors were scaled as described in Fig. 2. Relative positions of the profiles were established by making the  $H_2O$  and 40%  $D_2O$  profiles coincide at the centre of the membrane, assuming no water penetration at that point. Up to 10% water penetration at the centre of the membrane changes the scale negligibly. The  $H_2O$  and 40%  $D_2O$  water levels were then used to define the absolute density scale. The other profiles were then set on this scale without further adjustment. The assumption of a carbohydrate content in the cytoplasm of 20 g 100 ml<sup>-1</sup> (ref. 15) shifts the scale slightly. Protein and lipid headgroups have neutron scattering densities about equal to that of 40%  $D_2O$ , whereas lipid hydrocarbon chain scattering density is close to that of  $H_2O$ , increasing slightly with unsaturation. In 40%  $D_2O$ , the only component of the membrane contrasting with the Ringer's solution is the paraffin chain region. The small width observed (27.5 Å FWHM) of the density profile at this contrast confirms the presence of a lipid bilayer. This width is 60 Å in  $H_2O$ , and 32 Å in  $D_2O$ . *e*, Model density profile derived from egg lecithin bilayer neutron scattering data in  $H_2O$  (ref. 14), calculated for the same resolution and unit cell as the ROS data. The main difference between profiles *d* and *e* reflects the presence of rhodopsin in *d*, indicating that a considerable fraction of the protein must be inside the bilayer. *f*, Electron density profile of ROS (ref. 7), computed with ten orders, showing the polar headgroup positions. Computed with seven orders, it is identical to *c*. Density scales for *e* and *f* are arbitrary.

Fourier transform between orders 1 and 2, and tended to favour a positive sign for order 5 in  $D_2O$ . The phasing of orders 1-4 also agrees with that determined by Yeager in neutron diffraction experiments on intact retina<sup>13</sup>.

Neutron scattering density profiles of ROS computed with 7 orders are shown in Fig. 3, on an absolute density scale. The profile in  $H_2O$  (*d*) is markedly different from the neutron scattering density profile of phospholipid bilayers (*e*) and myelin<sup>14</sup> at the same resolution, in that it shows a fairly flat distribution of scattering density across the membrane instead of a pronounced dip at the centre. In the ROS, the scattering density at the centre of the membrane is matched



**Fig. 4** The broad in-plane diffraction is shown by this azimuthal scan of the diffraction intensity at a reciprocal spacing at about  $(50 \text{ Å})^{-1}$  from ROS in  $H_2O$  Ringer's solution. The origin of the angular scan is in the lamellar direction.

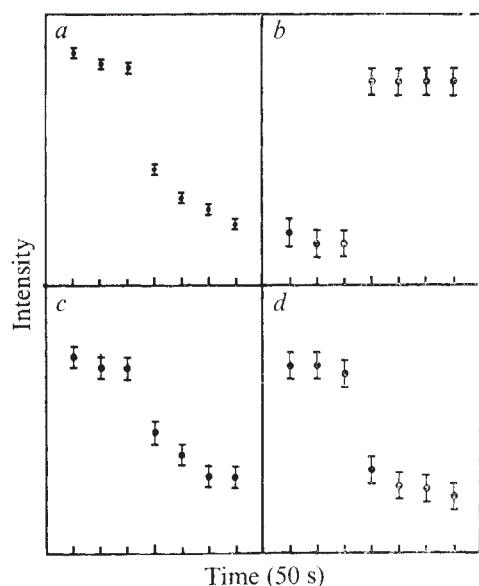
by the fluid medium when the Ringer's solution contains 15%  $D_2O$ . This means that the non-exchangeable material in the hydrophobic region has the scattering density of 15%  $D_2O$ , or 18%  $D_2O$  if one allows for 20 g carbohydrate per 100 ml cytoplasm<sup>15</sup>, independent of the amount of water penetration or proton exchange. Taking the average ROS fatty acid chain composition for frog ROS of 18:2 (calculated from ref 16), we find that about one-third of the volume in the hydrophobic region consists of protein. This could account for all of the protein. The water penetration in the membrane, assuming none at the centre, is given by the difference between the  $D_2O$  and  $H_2O$  profiles. From the variation in scattering density in the polar headgroup region (located by the electron density profile) we find that this region is about one-half water by volume.

## Equatorial diffraction

Diffraction in the direction perpendicular to the lamellar diffraction arises from interference between neighbouring rhodopsin molecules in the plane of the membrane. Diffuse equatorial diffraction centred near  $(50 \text{ Å})^{-1}$  has been observed in X-ray diffraction studies<sup>1,3,7</sup>. We measured in-plane diffraction in  $H_2O$  (Figs 1b and 4). It was much less clear in  $D_2O$  because of the very intense lamellar diffraction, and we could only estimate an upper limit for its intensity, which was about equal to the  $H_2O$  in-plane intensity. On an absolute scale, the equatorial intensity in 40%  $D_2O$  is within a factor of two of that in  $H_2O$ . These observations show that the in-plane intensity is insensitive to the scattering density of the fluid medium. Consequently it must primarily arise from protein-hydrocarbon chain contrast within the membrane. Allowing for uncertainties in these intensity measurements, we estimate that less than one-third of the protein volume is contrasting with the aqueous medium. The limit is mainly set by the measurement in 40%  $D_2O$ , where the protein-cytoplasm contrast is very small.

## Structural changes on bleaching

To investigate structural changes associated with total bleaching of rhodopsin, we recorded continuous sequences of spectra within a few minutes from dark-adapted samples



**Fig. 5** Intensity changes on bleaching for the first 4 orders of diffraction in 100%  $D_2O$ . Orders: *a*, 1; *b*, 2; *c*, 3; *d*, 4. The sequence of 50-s measurements was carried out on the same sample, which was exposed to light after the third measurement. Intensity changes amounted to 10% in order 1, 15% in 2, 7% in 3, and 10% in 4. Changes of comparable magnitude were observed in  $H_2O$  Ringer's solution, but the changes were much smaller in 40%  $D_2O$  Ringer's solution.

which were bleached in the middle of the sequences. In  $H_2O$  and  $D_2O$ , small but significant intensity changes were observed in the first 4 orders. In the weaker orders 5–7 the changes were too small to be measured in a short time. Bleaching had very little effect on the pattern in 40%  $D_2O$ . The bleaching sequence in  $D_2O$  Ringer's solution is shown in Fig. 5.

Since orders 1–4 are very intense at the above three contrasts, the small intensity changes observed on bleaching are most unlikely to be accompanied by phase changes. This is not the case with X rays, where order 2 is close to zero before bleaching and increases after bleaching, so that the difference profile is not uniquely defined. Neutron scattering density profiles in  $H_2O$  and  $D_2O$  and their changes on bleaching are shown in Fig. 6.

Although the differences between the profiles are smaller than the Fourier cutoff ripples, the differences are significant because they come from significant changes in the structure factors (Fig. 5). The observed effects are not accounted for by changes in  $d$  spacing, since X-ray diffraction experiments have shown that such changes in isolated rods are small and transient, with no net change in the time scale of our experiments (Fig. 2 of ref. 17). Furthermore, the minimal changes in 40%  $D_2O$  rule out a shift of the lipid bilayer within the unit cell. Protein, however, has the same scattering density as 40%  $D_2O$  and the observed changes on bleaching are consistent with an outward shift of protein at the cytoplasmic side of the membrane. Quantitatively, the observed change in the profile can be accounted for by a shift of the centre of gravity of the protein by about 1 Å.

## Conclusions

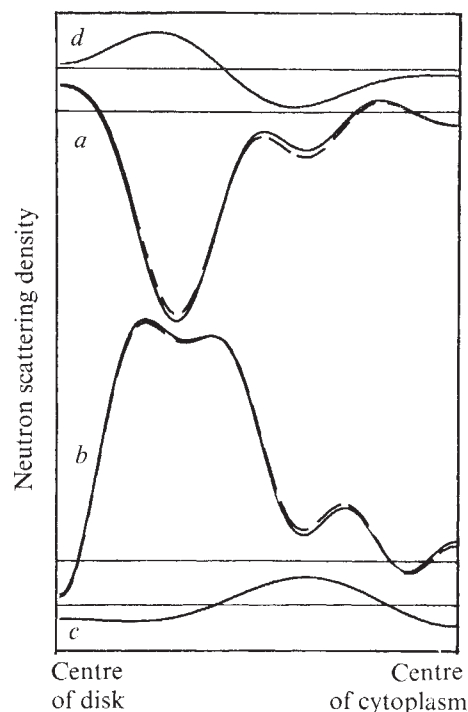
Our essential finding is that the shape of the ROS neutron scattering density profile in  $H_2O$  is not dominated by the bilayer arrangement of the lipids, as for the X-ray profile, and the contribution of protein to the structure is clearly seen. From the density profiles we calculate that a lower limit of 20% of the volume of the hydrophobic region consists of protein, allowing for uncertainty in the scattering

density level at the centre of the membrane due to Fourier cutoff effects and the possible presence of carbohydrate in the cytoplasm. This accounts for 50% of the total rhodopsin. Our upper limit locates all the rhodopsin in the hydrophobic region, which would then contain 35% protein by volume. The profiles also indicate a slight asymmetry in the membrane, with protein probably extending into the cytoplasmic space, but not into the intradisk space.

Since contrast variation was done without adding molecules which could be excluded from regions of hydrated protein, like sucrose, but merely by isotopic exchange of water, dry protein mass was observed. The part of the protein which extends outside the hydrocarbon chain region could be highly hydrated. There is definitely a large water penetration in the lipid headgroup region.

Insensitivity of equatorial diffraction to the scattering density of the Ringer's solution confirms that most of the protein is inside the membrane. These results on ROS membrane structure show that the electron density profile obtained from X-ray diffraction<sup>1–7</sup> should be interpreted as the electron density variation of the lipid bilayer, superimposed on a fairly uniform protein contribution.

The changes observed on bleaching of rhodopsin in our experiments agree with the X-ray results<sup>4,5,7</sup>. Furthermore, the contrast variation results identify the observed change as an outward shift of protein at the cytoplasmic side of the membrane. It cannot be decided whether this is due to a very small shift of the entire protein molecule or to a larger displacement of a small part of the protein. It seems that the only other evidence for a structural change in the intact membrane comes from small birefringence changes on bleaching<sup>15</sup>, and, more specifically, for the protein in the



**Fig. 6** Scattering density profiles of ROS disks before (—) and after (---) bleaching, and their difference profiles ( $\times 5$ ), (bleached — unbleached). Changes in orders 1–4 have been included in this computation. Changes in orders 5–7 are smaller and are not included in the difference profiles. The  $H_2O$  and  $D_2O$  data are on different density scales. *a*, In  $D_2O$  Ringer's solution. *b*, in  $H_2O$  Ringer's solution; *c*,  $H_2O$  difference profile ( $\times 5$ ); *d*,  $D_2O$  difference profile ( $\times 5$ ). The increase in density at the intradisk side in the  $D_2O$  profile may be accounted for by a shift of  $D_2O$  into that region, resulting from increased hydrogen-deuterium exchange on bleached rhodopsin<sup>18</sup>.

membrane, from the observation of changes in the hydrogen exchange kinetics on bleaching<sup>18</sup>.

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# letters to nature

## On being close to a black hole without falling in

I SHOW here that for any  $\varepsilon > 0$  it is possible for a magnetic field to hold plasma in equilibrium at a radius  $r_0 = r_g(1 + \varepsilon)$  relative to a Schwarzschild black hole of (radius  $r_g$ ) and that this equilibrium need not be subject to the kind of relativistic instability that destroys circular orbits of radius  $r < 3r_g$ . (Putting  $c = G = 1$ ,  $r_g = 2M$  is the radius of the event horizon of a hole of mass  $M$ .) Astrophysical plausibility does not stop  $\varepsilon$  from being much smaller than unity.

Normal indices refer to Schwarzschild coordinates, whereas barred indices refer to the corresponding orthonormal tetrad and label physical quantities which can be measured by observers at rest relative to the hole. Suppose there is a thin disk of matter at rest in the  $\theta = \pi/2$  plane with current density  $J_{\bar{\phi}}$  and energy density  $\rho$  as measured by an observer at rest in the disk. The current is to interact with a magnetic field  $B_{\bar{\theta}}$  to produce a Lorentz force balancing gravity. I shall assume that both the matter and the field have negligible gravitational effect, so that the Schwarzschild metric is valid. Then

$$T^{\bar{r}\bar{\theta}}_{;\bar{\theta}} = (\rho + p)M/r^2 + \partial p/\partial r - 2p\left(1 - \frac{2M}{r}\right)/r$$

Here  $p$  is the gas pressure. I shall assume that the plasma is not highly relativistic, so that  $p \ll \rho$  and  $T^{\bar{r}\bar{\theta}}_{;\bar{\theta}} = \rho M/r^2$ . Taking the radial component of the equation of motion  $T^{\bar{a}\bar{b}}_{;\bar{b}} = F^{\bar{a}\bar{b}}J_{\bar{b}}$  and integrating with  $r d\theta$ , I obtain

$$\begin{aligned} \sigma M/r^2 = F^{\bar{r}\bar{\theta}}I_{\bar{\phi}} &= (1 - (2M/r))^{\frac{1}{2}} F^{\bar{r}\bar{\theta}}\bar{I}_{\bar{\phi}} \\ &= (1 - (2M/r))^{\frac{1}{2}} (-B_{\bar{\theta}})I_{\bar{\phi}} \end{aligned} \quad (1)$$

where  $\sigma$  and  $I_{\bar{\phi}}$  are the energy and current surface densities.

Suppose for simplicity that the magnetic field is mainly generated far from the hole so that near the hole it seems to be uniform. Suppose this 'uniform' field has magnitude  $B_{\infty}$  and points in the  $\theta = 0$  direction at infinity. Then  $B_{\bar{\theta}} = -(1 - (2M/r))^{\frac{1}{2}} B_{\infty}$  on  $\theta = \pi/2$  (ref. 1). The field generated by the local current is of the order  $(1 - (2M/r))^{\frac{1}{2}} I_{\bar{\phi}}$  except where  $I_{\bar{\phi}}(r)$  varies rapidly, for example at the edge of a current disk<sup>2</sup>. For consistency it should be assumed that  $I_{\bar{\phi}}(r) \ll B_{\infty}$  and varies smoothly. This is not an important restriction as boundary conditions at the event horizon require  $B_{\bar{\theta}} \approx B_{\infty}(1 - (2M/r))^{\frac{1}{2}}$  close to the event horizon,  $B_{\infty}$  being a constant<sup>3</sup>, and the only place one might expect  $I_{\bar{\phi}}$  to be discontinuous is at the inner edge of the disk, where the effect would be to make  $B_{\bar{\theta}}$  more

negative, and thus to oppose gravity. Substituting the assumed form for  $B_{\bar{\theta}}$  into equation (1)

$$(\sigma/I_{\bar{\phi}})M = (r^2 - 2Mr)B_{\infty} \quad (2)$$

Let  $\sigma/I_{\bar{\phi}} = \alpha(m_p/c)$ , where  $m_p$  and  $e$  are the mass and charge of a proton.  $m_p/c$  is the dimensionless number  $8.99 \times 10^{-19}$  when  $c = G = 1$ . Clearly  $\alpha > 1$ . If the electrons and protons are streaming relative to each other with a relativistic velocity, their kinetic energy contributes to  $\sigma$ , and if two-stream instability is to be avoided the plasma must be relativistically hot, which also increases  $\sigma$ . The relativistic current would cause a significant mechanical stress  $T^{\phi\phi}$ , which because of the curvature of the coordinate system would contribute to the radial balance equation as a force opposing gravity; this is just the centrifugal force from the circular motion of the particles. I shall assume that the current is non-relativistic (so that  $T^{\phi\phi}$  is not significant near the event horizon) and take  $\alpha \gtrsim 10$ . Fixing  $\alpha$  at its lower limit in equation (2) and solving for  $r$ , I obtain  $r_0$ , the smallest radius at which matter can be stationary, given  $B_{\infty}$  and  $M$

$$\begin{aligned} r_0/r_g &= (1 + (1 + 4\varepsilon)^{1/2})/2. \\ \varepsilon &= 5.3\alpha(B_{\infty}/1 \text{ gauss})^{-1}(M/M_{\odot})^{-1} \end{aligned} \quad (3)$$

If  $\varepsilon \ll 1$ ,  $r_0/r_g \approx 1 + \varepsilon$ . If the field is a significant gravitational source  $B_{\infty} \gtrsim 10^{19}(M/M_{\odot})^{-1}$  gauss. Hence  $B_{\infty}$  can be large enough for  $\varepsilon \ll 1$  without being so large that the Schwarzschild metric is invalid.

This result does not contradict Gibbons' limit on how near to a black hole a rope can be lowered<sup>3</sup>. Gibbons' rope has constant energy density per unit length. By the weak energy condition this gives an upper limit on the tension the rope can take without breaking. Tension is greatest at the top end of the rope, but gravity exerts its greatest force on the energy present at the bottom of the rope. The rope breaks at the top. This can be avoided by strengthening the rope at the top and lightening it at the bottom, for example by lowering a tapered rope. This is effectively happening here. The magnetic field can be regarded as a medium with pressures and stresses. The energy in a spherical shell of radius  $r$  and unit thickness  $\sim r^2 B_{\infty}^2$ . There is only a small amount of energy near the hole requiring support, and a lot of energy to take the strain far away from the hole. Hence equilibrium is possible.

It only remains to consider stability. Distant field sources can be expected to vary on time scales much longer than the free-fall time near the hole. I shall therefore take the uniform, externally imposed part of the field to be constant. Because of high



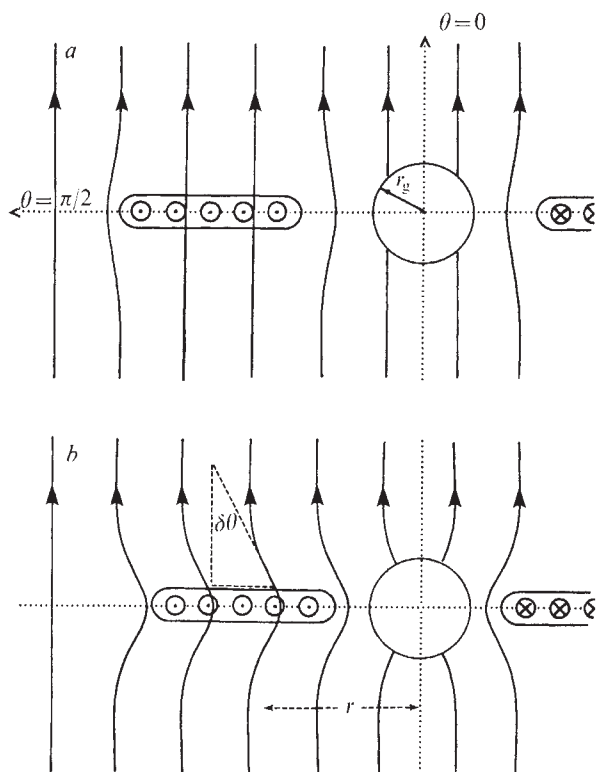


Fig. 1 *a*, Cross section of current disk and black hole in unperturbed state. Large circle is the event horizon of the hole. Small circles with dots denote current coming out of the paper etc. The magnetic field lines are lines of constant  $A_\phi \approx (B_\infty/2)r^2 \sin^2 \theta$  *b*, Perturbed state. Field lines are frozen into the disk and at infinity. A field line through an element of the disk displaced a radial distance  $\delta r$  will be similarly displaced. At the disk surface the line is rotated through an angle  $\delta \theta$  as seen on this diagram—a local observer would see it rotated through an angle

$$\bar{\delta \theta} = \delta \theta (1 - (r_g/r))^{-1/2}$$

conductivity, any perturbation of the plasma drags the field lines with it. The perturbed field consists of the original uniform field and a perturbation field generated by currents in the plasma. For a given perturbation, the electromagnetic energy increases by an amount proportional to  $B_\infty^2$ , while the decrease in gravitational potential energy is fixed. Thus the perturbation is stable if  $B_\infty$  is large enough. Equation (1) yields

$$\left( \frac{\sigma}{-B_\phi} \right) M = r^2 (1 - (2M/r))^{1/2} I_\phi^- \quad (4)$$

Consider a perturbation (not necessarily axisymmetric) that takes a fluid element from  $r$  to  $r + \delta r$ , and does not extend over a length scale  $\gg r$ . For that element the left-hand side of equation (4) stays constant, because surface energy and flux satisfy identical conservation laws. The perturbation is stable if, and only if, the right-hand side increases. When unperturbed the field lines (from a static observer's viewpoint) lines of constant covariant vector potential  $A_\phi(r, \theta)$  are nearly straight (Fig. 1*a*). Because the perturbation field is approximately dipolar at heights  $\gg r$  above the disk the field lines there will also be almost straight (Fig. 1*b*). This gives a lower limit on the angle  $\delta \theta$  through which the field lines have been rotated near the disk:  $\delta \theta \gtrsim (-\delta r)/r$ , and implies  $\delta I_\phi^- \gtrsim B_\infty (-\delta r)/r$ . Close to the hole the disk would seem to be stable if  $I_\phi^- \lesssim 2B_\infty (1 - (2M/r))^{1/2}$ .

For a stellar black hole  $M \approx 10 M_\odot$ , while for a black hole in a galactic nucleus or QSO  $M \approx (10^8 - 10^9) M_\odot$ . Typical interstellar fields have magnitudes of only  $\sim 10^{-5}$  gauss, but fields frozen into accreting plasma get amplified by many orders of mag-

nitude<sup>4</sup>, so that  $B_\infty$  is in fact much greater. Small scale fields dissipate faster than large scale fields. It is possible that plasma is in quasi-static equilibrium near to some black holes. This is a consequence of  $m_p/c\epsilon$  being a small number, that is, of the strength of electromagnetic forces compared with gravity.

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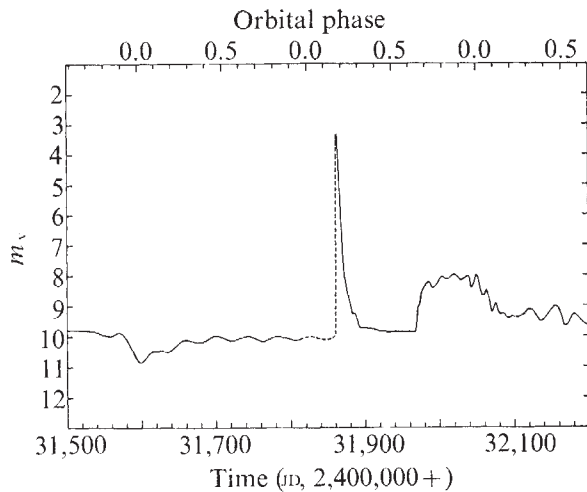
## Outbursts of the recurrent nova T Coronae Borealis

THE recurrent nova T CrB has long been regarded as an anomaly among cataclysmic variables. It is a binary system consisting of an M3III giant and a blue companion<sup>1</sup>. From the radial velocity curves, Kraft<sup>2</sup> found component masses of  $\geq 3.7 M_\odot$  and  $\geq 2.6 M_\odot$ , respectively, later revised downwards to  $2.6 M_\odot$  and  $1.9 M_\odot$  by Paczyński<sup>3</sup>. Nevertheless, the mass of the blue component is clearly greater than the Chandrasekhar limit, even though the blue components of common novae are widely believed to be white dwarfs. The binary period, 227.5 d, is  $> 300$  times longer than that of GK Per<sup>3</sup>, the longest period known among the remaining cataclysmic variables, and in stark contrast with the 0.0567-d period of WZ Sge, the only other recurrent nova of known binary period. Warner<sup>4</sup> has suggested, however, that T CrB is typical of the majority of recurrent novae. In this paper we interpret the behaviour of T CrB in terms of accretion in a binary system.

Two outbursts of T CrB have been observed, in 1866 (JD 2,402,734) and in 1946 (JD 2,431,861), these being apparently remarkably similar<sup>5</sup>. In neither case was the eruption detected before the nova was already declining in brightness, but evidence exists suggesting that the initial rise is extraordinarily rapid<sup>6</sup>.

The brightness variations in this system in the interval immediately surrounding the 1946 outburst<sup>7,8</sup> are shown in Fig. 1. Three features are clearly visible in the light curve: (1) There is a sudden decrease in brightness by about one magnitude (visual) to a minimum,  $m_v \approx 10.8$ , at JD 2,431,599. What scattered observations exist of this phenomenon suggest that superimposed on the recovery from minimum is a decaying oscillation with period  $\sim 41$  d and initial amplitude,  $\Delta m_v \approx 0.15$ ; (2) 263 d later, the principal maximum,  $m_v \approx 3.4$ , occurs. The system remains within one magnitude of maximum for approximately three days only, and the decline is exceptionally rapid ( $0.5 \text{ mag d}^{-1}$ ). The total energy output in visual light during this outburst amount to  $\sim 1,400$  times the mean daily output of the giant component<sup>5</sup> ( $m_v \approx 10.2$ ) in a quiescent state; (3) 106 d following the principal maximum, the system again begins brightening, reaching a secondary maximum,  $m_v \approx 8.06$ , near JD 2,432,020, with fluctuations at maximum of a few tenths of a magnitude. The system drops to  $m_v \approx 9.4$  within the next 80 d, followed by an extremely prolonged decline<sup>9</sup> ( $\geq 25$  yr) to its present light level,  $m_v \approx 10.15$  (personal communication from J. A. Mattei).

This outburst behaviour carries the distinct signature of an episodic accretion event. The minimum preceding outburst is associated with the dynamical ejection of a coherent mass of gas by the lobe-filling giant. Bath<sup>10</sup> has shown the existence of such an instability generally among lobe-filling giants, and



**Fig. 1** The light curve of T CrB during the 1946 outburst, reconstructed from visual observations by members of the AAVSO (ref. 7). The dotted portion of the curve near JD 2,431,840 represents a gap in observational records while T CrB was unfavourably located with respect to the Sun. The observations shown before the outburst are those of a single observer, L. C. Peltier. The oscillations shown in this portion of the light curve are suggested by his observations, but the number of observations is relatively small. There is unusually large observational scatter after JD 2,432,070, suggesting an enhanced level of rapid photometric activity. The orbital phases indicated at the top of the figure are computed from an ephemeris by Paczyński<sup>3</sup>.

time-dependent calculations of such events find the mass-losing star underluminous following the ejection<sup>11</sup>. The recovery from minimum is characterised by a timescale<sup>11</sup>

$$\tau_{th} \approx \frac{RT\Delta M}{\Delta L}$$

where  $R$  is the ideal gas constant. A lobe-filling M3III giant<sup>12</sup>,  $T_e = 3,350$  K, has a luminosity  $L_g = 1,250 L_\odot$ . Taking  $\Delta L \approx L_g$ , and assuming a mean temperature,  $T \approx 10^6$  K, for the ionisation zones, the observed timescale,  $\tau_{th} \approx 100$  d, indicates  $\Delta M \approx 10^{-3} M_\odot$  transferred. The possible 41-d periodicity superimposed on the recovery is consistent with a low order pulsational mode of the giant (P. R. Wood, personal communication), excited by the ejection episode.

The principal maximum is of such short duration that it must correspond to a dynamical phenomenon. The matter ejected by the giant initially takes up an extremely eccentric orbit around the secondary. This orbit is made circular dynamically, by supersonic collisions within the orbiting material. A simple estimate, following Lubow and Shu<sup>13</sup>, indicates that  $1.09 \times 10^{14}$  erg g<sup>-1</sup> is dissipated in this way for the adopted binary parameters. Allowing a bolometric correction of 1.81 mag for the giant<sup>12</sup>, the visual energy output is found to be  $1.12 \times 10^{44}$  erg by comparison with the quiescent giant luminosity, giving  $\Delta M = 5.2 \times 10^{-4} M_\odot$ , in good agreement with the estimate obtained above. The resultant circular orbit has a radius  $R_d = 19.9 R_\odot$ , period 7.49 d.

Viscous dissipation within this accretion ring broadens it into a disk which gradually reaches down to the surface of the accreting star<sup>14</sup>. As it does so, its luminosity increases, reaching a maximum as its inner edge reaches the surface of the central star. The height of that maximum is a direct measure of the surface potential of the star, and the interval between the principal and secondary maxima reveals the viscous timescale of the disk.

The secondary maximum of T CrB clearly reveals the secondary as a main-sequence star (first suggested by Plavec<sup>15,16</sup> and Harmanec<sup>17</sup>). The mass and luminosity of the giant component indicate an age<sup>18</sup> of  $\sim 4.7 \times 10^8$  yr for the system, placing the main-sequence companion in core hydrogen

burning ( $X_c \approx 0.28$ ), with radius  $R \approx 1.81 R_\odot$ . In the time-dependent, constant-viscosity disk model<sup>14</sup>, the interval between the creation of an accretion ring (primary maximum) and the point at which the light level rises to within 1.5 mag of secondary maximum (an observationally well defined quantity) is  $\Delta t = 0.118 \tau_v$  where  $\tau_v$  is the natural viscous timescale

$$\tau_v = \frac{R_d^2}{6\nu}$$

Thus, the viscous timescale of the accretion disk in T CrB is  $\tau_v = 930$  d. The predicted height of secondary maximum for the assumed main-sequence companion is  $m_v = 7.77$ , in good agreement with the observed maximum (8.06 m), and far lower (by more than five magnitudes) than that expected for a massive white dwarf companion. The effective Reynolds number of the disk is

$$R_v \approx 6 \left\{ \frac{GM_2}{R_d^3} \right\}^{1/2} \tau_v \approx 4,700$$

indicating that the disk is turbulent.

Dwarf novae are widely believed to be accretion episodes of a very similar kind<sup>19,20</sup>. Bailey<sup>21</sup> has recently found an empirical relationship among dwarf novae between the rate of decline from maximum and the orbital period,  $P$

$$\tau \approx 9.2P$$

where  $\tau$  is the time required to decline one magnitude. For T CrB, the inferred timescale,  $\tau = 2,090$  d, is in excellent agreement with the time required for a theoretical constant-viscosity disk with  $\tau_v = 930$  d to decline from one magnitude below maximum to two magnitudes below maximum,  $\Delta t = 2,320$  d.

Details of this model, and a reinterpretation of Kraft's observations<sup>9</sup> will be published separately. The author gratefully acknowledges invaluable discussions with J. W. Truran, R. P. Kraft, G. T. Bath, J. E. Pringle, and P. R. Wood. Special credit is due to the many amateur astronomers throughout the world, and the American Association of Variable Star Observers (AAVSO) and its Director, Janet Mattei, in particular. Without their patient observations this discovery would not have been possible. This research was supported by the NSF.

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## A plum-pudding model for interstellar grains

THERE seem to be two basic mechanisms by which interstellar grains can grow; the condensation of gaseous molecules (mostly of the CNO type) on to their surfaces and the coagulation on collision of individual grains. In general, as has been shown by Simons and Williams<sup>1</sup>, condensation leads to a more rapid growth rate than coagulation resulting from the thermal motion of the grains. If, however, relative motions can be set up within the grain population, then this increases the collision rate and so also increases the rate of growth by coagulation. Now, for small grains, the force on them from radiation pressure depends on a different function of the grain radius,  $a$ , than does the viscous drag generated by the ambient gas. (For simple models these functions are respectively  $a^2$  and  $a^3$ .) Consequently, grains of differing sizes will be caused to move with different velocities through the ambient gas by radiation, the larger ones moving faster. This causes these grains to capture further grains by coagulation which in turn enhances their relative velocity even further. The purpose of the present communication is to show that this growth caused by radiation pressure coagulation is probably of the same order as that from gas atom condensation, and to point out an important consequence of this.

Let us focus attention on a particular grain, G. Atoms will be incident on this grain both in the form of free atoms and in the form of those bound in other grains. In each case the number of atoms  $Z$  adhering to G per unit time will be given by

$$Z = vnA\sigma$$

where  $v$  is the velocity of the incident atoms relative to G,  $n$  is the number of these atoms per unit volume,  $A$  is the effective cross section of G and  $\sigma$  is the sticking coefficient. Thus the ratio of grain growth from condensation to that caused by radiation pressure coagulation is given by

$$X \simeq \frac{v_a n_a A_a \sigma_a}{v_g n_g A_g \sigma_g} \quad (1)$$

where the subscripts a and g refer, respectively, to free atoms and to those bound in grains.

To calculate  $v_g$  we note first that due to its charge, prolonged grain motion can only occur parallel to the magnetic field  $\mathbf{H}$ . In this direction, the force on grains of radius  $a$  from radiation is given by

$$F_R = \pi a^2 Q(a) P \beta \quad (2)$$

where  $P$  is the radiation pressure and  $\beta$  the cosine of the angle between  $\mathbf{H}$  and the direction of the radiation pressure.  $Q(a)$  is the mean efficiency factor for radiation averaged over all frequencies. For blackbody radiation characterised by a temperature of  $\sim 10^4$  K, such as may typically be found in the Galaxy, acting on a grain composed of material with a complex refractive index

$$Q(a) \simeq 5 \times 10^4 a \quad (3)$$

(see Simons and Williams<sup>2</sup>, Greenberg<sup>3</sup>), for all values of  $a$  such that  $Q(a) \leq 1$ . Simons and Williams<sup>2</sup> have shown that for the general galactic radiation field

$$P \simeq 1.2 \times 10^{-13} \text{ (cgs units)}$$

and that the gravitational field of the interstellar cloud within which the grain is situated is unimportant as far as the motion of that grain is concerned. Also since the galactic field acts

equally on grain and cloud, in any discussion of their relative motion it may be ignored. The relative velocity of the grain is thus determined by balancing the driving radiation pressure by the viscous drag of the ambient gas which has the form (Baines *et al.*<sup>4</sup>)

$$F_{\text{Res}} = \pi a^2 \rho v W \gamma \quad (4)$$

$v$  being the relative velocity,  $W$  the mean thermal velocity and  $\rho$  the gas density.  $\gamma$  is a factor, numerically of order unity, which depends on the way gas molecules are reflected from the surface of the grain. For typical interstellar clouds (see Lequeux<sup>5</sup>) we find that

$$F_{\text{Res}} \simeq 4.5 \times 10^{-18} \pi v a^2 \quad (5)$$

and from equations (2) and (5) we get

$$v \simeq 1.3 \times 10^4 Q(a) \quad (6)$$

taking  $\beta = \frac{1}{2}$ . Thus the relative velocity of two species of grain of radii  $a_1$  and  $a_2$  is given by

$$v = 7 \times 10^8 (a_1 - a_2) \quad (7)$$

the larger grains moving faster than the smaller ones. Since we are concerned with the growth of grains to their present size we take as representative values  $a_1 \simeq 4 \times 10^{-6}$  cm and  $a_2 \simeq a_1/2$ . This yields

$$v_g \simeq 15 \text{ m s}^{-1} \quad (8)$$

As far as  $v_a$  is concerned, we suppose the atoms to be predominantly oxygen and the gas temperature to be  $\sim 100$  K. This gives  $v_a \simeq 300 \text{ m s}^{-1}$ , so that

$$v_a/v_g \simeq 20 \quad (9)$$

As far as  $n_a/n_g$  is concerned, it is generally assumed that the mass of heavy condensable atoms is roughly equally divided between free atoms and those bound in grains. In view of this we shall take

$$n_a/n_g \simeq 1 \quad (10)$$

It is generally supposed that atoms such as C and Si are predominantly bound in grains so that the ratio  $n_a/n_g$  may well be  $< 1$ , though values  $> 1$  cannot be entirely ruled out.

$A_a$  will be the geometric cross section  $\pi a^2$  of the grain G with radius  $a$ . If the other grains incident on it have radius  $a'$ , then

$$A_g \simeq \pi(a+a')^2$$

Now  $a'$  can be both greater or smaller than  $a$ , so that a reasonable estimate is presumably made by setting  $a' = a$ . This gives

$$A_a/A_g \simeq 1/4 \quad (11)$$

The value of  $\sigma_a$  has been estimated theoretically for O atoms (in ref. 6) to be  $\sigma_a \simeq 0.3$ . The best available evidence for  $\sigma_g$  would seem to be from experiments on the acoustic coagulation of terrestrial aerosols<sup>7</sup>. In these experiments coagulation proceeded efficiently for all relative grain velocities involved, which ranged up to several  $\text{m s}^{-1}$ . Since  $v_g$  (equation (8)) is greater than these velocities only by a factor  $\sim 3$ , we suggest taking

$$\sigma_g \simeq 1$$

in the absence of any evidence to the contrary. This gives

$$\sigma_a/\sigma_g \simeq 0.3 \quad (12)$$



It then follows from equations (1), (9), (10), (11), (12) that

$$X \approx 2 \quad (13)$$

In spite of the various uncertainties in the above estimation the result (equation (13)) would seem to substantiate our contention that growth from radiation pressure coagulation is probably of the same order as that from condensation. This has a very important consequence since it leads to the possibility of the formation of a grain possessing a novel type of internal structure; we call this a "plum-pudding" grain.

Initially a grain with a single silicate type nucleus will build up an ice mantle by condensation. Collision with a similar grain followed by coagulation will lead to a larger grain consisting of two separate silicate type cores surrounded by ice. As growth by both mechanisms proceeds, a multicored grain of silicates and similar material embedded in an ice matrix is formed, similar to plums in a pudding.

Such a grain would have rather novel optical characteristics, the main feature being that it would, at one and the same time, exhibit structure at two characteristic lengths, that of the nuclei and that of the whole assembly, including the ice. One might speculate that with such a grain it would be possible to explain the optical observations which according to Whittet *et al.*<sup>8</sup> have hitherto required the existence of three distinct grain models. Speculating further, the requirement of large grains, pointed out by Rowan-Robinson<sup>9</sup>, might also be met by our type of grain structure.

The above treatment would seem to demonstrate the plausibility of the existence of plum-pudding grains. A quantitative treatment of their growth, structure, size and optical properties remains, however, to be performed. Work on this is currently in progress and will be published elsewhere.

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## A comparison of acoustic Doppler vertical velocities with direct measurements in the atmospheric boundary layer

THE quantitative evaluation of the intensity of acoustic echoes from the first few hundred metres of the atmospheric boundary layer (in both stable and convective situations) has been the subject of much recent experimental work<sup>1-3</sup>. The acoustic echo, however, contains Doppler information on the vertical air motion and this could prove of great use in the specification of further important boundary layer statistics such as  $\sigma_w^2$ , the variance of the vertical wind speed<sup>4</sup> and  $\epsilon$ , the rate of dissipation of turbulent kinetic energy. Beran *et al.*<sup>7</sup> using a sounder in the monostatic mode (transmitter and receiver aeri- als located together) derived the profile of the vertical wind component using the acoustic Doppler technique. They did not, however, verify that the estimates of the winds were

correct, nor did they record the magnitudes of the Doppler shifts in the weak echo regions between the periods of thermal activity. This paper presents a quantitative comparison between acoustic Doppler vertical winds and direct estimates measured at a fixed height in the atmosphere.

The acoustic sounder operates at a frequency of 1,730 Hz transmitting pulses of length 74 ms at a pulse repetition frequency of 0.44 Hz and up to 25 W of acoustic power. The aerial (which is used for both transmission and reception) is a  $6 \times 6$  array of 16.5-cm diameter re-entrant horn loudspeakers. These figures correspond to a height range of 380 m and a range resolution of 12.25 m. The pulse length (and hence the resolution) and the transmitted power can be varied. The receiver gain can be varied up to 155 dB which includes a ramp gain to compensate for the spherical divergence of the acoustic echoes as they are received from the longer ranges<sup>8</sup>. The transmit pulse is used to synchronise a gating system which controls the selection of the signal received from any chosen height for Doppler shift determination. The gating system provides five gates of variable width hence allowing the signal to be analysed at five different heights. A Hewlett-Packard spectrum analyser with an 8553B r.f. section is used to produce the frequency spectrum of the received echo. The oscilloscope display is automatically photographed using a cine camera triggered by the same gating circuit. The photographed power spectra can be analysed subsequently to determine the frequency shift by use of an expression for the radial velocity  $v$  of a scattering eddy given by Beran *et al.*<sup>7</sup>

$$v = \frac{C}{2} \left( \frac{f_0}{f_d} - 1 \right)$$

for relatively low velocities of up to  $2 \text{ m s}^{-1}$ . Here  $f_0$  is the transmitted carrier frequency and  $f_d$  is the frequency of the scattered signal.

Direct estimates of the vertical velocity were made using a 'reduced' version of the turbulence probe described by Readings and Butler<sup>9</sup>. Basically this instrument consists of a set of sensors mounted on a vane which keeps them facing into wind. The vane is attached to the tethering cable of a large kite balloon ( $\sim 1,300 \text{ m}^3$  volume) and is free to move in the horizontal plane. In this particular application the sensors consisted of a hot wire (double 'V' shaped) inclinometer mounted on a damped pendulum at the front of the vane and a multislot photoelectric anemometer fitted with a 6-cup polystyrene rotor. These sensors record the inclination ( $\phi$ ) of the wind vector to the local horizontal (as seen by the sensor) and the total wind speed,  $V$ . Thus the instrumentation does not precisely measure the absolute magnitude of the vertical velocity but rather its variability about some chosen mean. The mean value is set by assuming that the average inclination of the flow is zero,  $\bar{\phi} = 0$ , where the double bar represents the complete period of a run (typically  $\sim 1 \text{ h}$ ). Average vertical velocities ( $\bar{w}$ ) over time periods corresponding to fifty sounder pulses are then obtained from the relationship

$$\bar{w} = V \sin(\bar{\phi} - \bar{\phi})$$

Several experiments have recently been carried out to determine the overall accuracy of the balloon-borne turbulence instrumentation including the sensitivity of the observations to sensor design, frequency response, data reduction techniques and the influence of probe motion as it sways with the balloon cable (see Readings and Butler<sup>9</sup>, Haugen *et al.*<sup>10</sup>). The results show that the tolerance on the vertical velocity estimates is  $\sim 5\%$  and that this wind component is negligibly affected by balloon cable movement.

The results described concern four experiments carried out in convective conditions and two in stable conditions. Figure 1a

shows the facsimile recording for one of the runs in convective conditions on June 18, 1975. The sequence of thermal activity is clearly shown. During this run the turbulence probe, providing the direct measurements, was located at a height of 70 m. Figure 1b shows the comparison between vertical velocities as recorded by the sounder and the probe, the latter being averaged over a time interval corresponding to twenty sounder pulses that is, 46 s, which for the wind speed present during this run is equivalent to a spatial scale of  $\sim 200$  m. This distance is much greater than the separation of probe and sounder scattering volume (80 m). Sufficient signal was still received during periods of weak echo to record a Doppler shift, provided the gain of the receiver was sufficiently high. For both convective and stable conditions the received signal was too weak to permit the recording of the Doppler spectrum on  $\sim 5\%$  of the total number of occasions. Comparison of Fig. 1a and b shows that there is little apparent connection between the intensity of the echo ( $C_T^2$ ) and the updraughts as seen by both sounder and turbulence probes. Beran *et al.*<sup>7</sup> have, however, observed that areas of maximum  $C_T^2$  were associated with regions of maximum vertical wind shear. This is of importance in the qualitative interpretation of acoustic sounder facsimile records and merits further study.

Figure 2 shows a plot of vertical velocity estimates from the acoustic Doppler measurements averaged over fifty pulses against direct measurements over a corresponding time interval using data from all four runs in convective conditions. The data are seen to scatter fairly well about a line of unit slope. A best fit line through the data has slope 0.75 and intercept  $0.2 \text{ m s}^{-1}$  on the sounder axis. It is not clear to what extent the scatter in this plot is produced by real spatial differences in the vertical velocity field or by the inherent errors in the technique. It should be emphasised that the probe provides a continuous estimate of the vertical velocity at one point only whereas the sounder estimates refer to a discrete set of observations averaged in height (over a resolution depth) and in the horizontal (over the beam area). The values of the correlation coefficients for unstable conditions ranged between 0.7 and 0.9 and the standard deviations of the direct and indirect estimates of vertical velocity were between  $0.43$  and  $0.53 \text{ m s}^{-1}$  and  $0.4$  and  $0.6 \text{ m s}^{-1}$  respectively. Figure 3 shows the facsimile recording and the plot of vertical velocity against time obtained during stable conditions on June 26, 1975. For these conditions the correlation coefficients were 0.7 and 0.9 with direct and indirect

Fig. 1 a, Facsimile recording of convective conditions between 10.33 and 11.20 on June 18, 1975. b, Comparison between sounder estimates (○) and direct measurements (●) of vertical velocities averaged over twenty pulses.

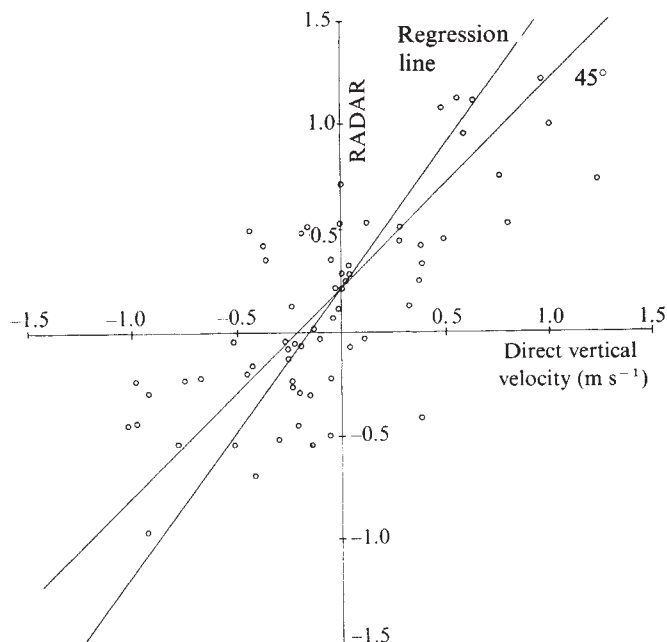
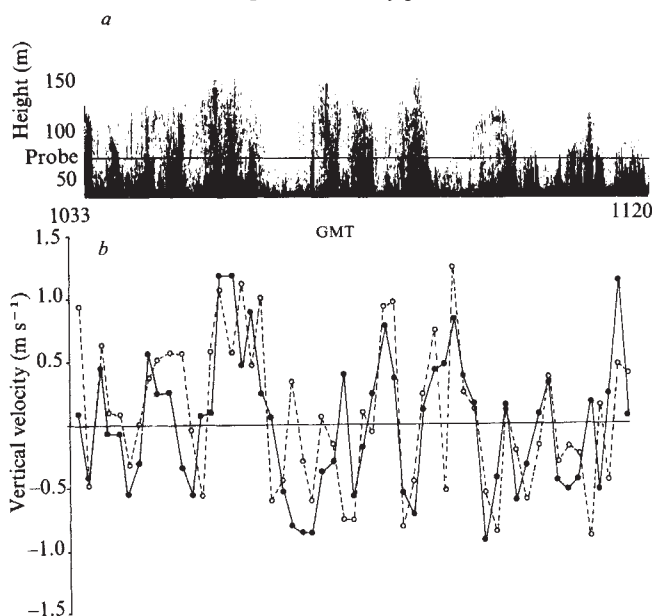


Fig. 2 Plot of vertical velocity estimates from the acoustic Doppler measurements against the direct measurements from all four experiments in convective conditions.

standard deviations of  $0.06$  and  $0.04 \text{ m s}^{-1}$  and  $0.1$  and  $0.06 \text{ m s}^{-1}$  respectively. The variances recorded refer to the frequency band set by the run duration and the averaging period.

Three sources of error indicated by Spizzichino<sup>11</sup> have been considered in some detail. These are (1) instrumental error; (2) refraction effects and; (3) apparent vertical velocities introduced by horizontal wind components because of the finite beamwidth of the acoustic antenna. The relationship that describes the instrumental error is

$$\sigma_w^2 = \frac{\sqrt{(3)\lambda^2 b_1^2}}{4N\tau_G S_1}$$

where  $S_1 = ((1/\tau_G^2) + b_1^2)^{1/2}$ ,  $N$  is the number of pulses,  $\tau_G$  is the actual width of the sampling gate and  $b_1$  is the bandwidth of the Doppler signal. For the present results  $b_1$  is  $\sim 10 \text{ Hz}$ ,  $\tau_G$  is  $180 \text{ ms}$  and  $N$  is  $50$ , giving a value for  $\sigma_w$  of  $0.12 \text{ m s}^{-1}$ , which is reasonably small. For  $N=5$ ,  $\sigma_w$  becomes  $0.4 \text{ m s}^{-1}$  and in accordance with this the scatter in a plot of direct against sounder estimates was much greater than that in Fig. 2 and such that no discernible trend could be identified. A further source of error is in the determination of the position of the peak of the power spectra, necessary for the measurement of the frequency shift. This error was estimated to be  $\sim 0.1 \text{ m s}^{-1}$  for a single spectral photograph.

The error from refraction can be found if the integrated wind profile to the height of the observation is known. During these experiments this was obtained using the Cardington Balthum<sup>12</sup> and this error turned out to be  $\sim 0.2 \text{ m s}^{-1}$ . This indicates a systematic overestimation of the vertical velocity by the sounder and may be responsible for the positive intercept of the regression line on the sounder axis (see Fig. 2).

The error from the finite antenna beamwidth ( $\sim 10^\circ$ ) is more difficult to assess since it depends on whether the observation time is long enough for the reflectivity being considered to be statistically uniform in the scattering volume. If this is the case then it merely produces spectral broadening and is already included in the instrumental error which involves  $b_1$ . If, however, the reflectivity is not uniform and there are many reflectivity maxima within the scattering volume, then the echo spectrum will also exhibit subsidiary maxima. Such

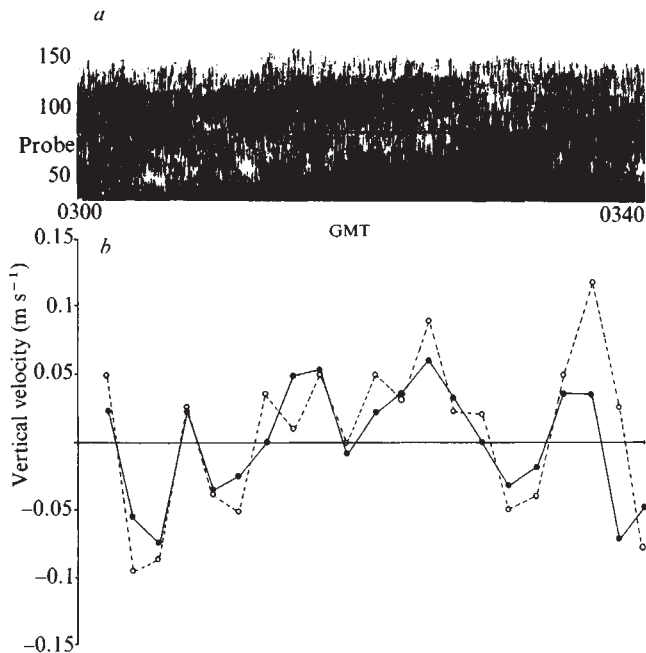


Fig. 3 a, Facsimile recording of an inversion between 03.00 and 03.40 on June 26, 1975. b, Comparison between sounder estimates (○) and direct measurements (●) of vertical velocities averaged over fifty pulses.

spectra were infrequently observed (~ 2% of the time) and hence for the data presented here this error can be neglected.

The results presented above show that reasonable estimates of the vertical wind velocity can be obtained from the returns of a monostatic acoustic sounder operating in both stable and convective atmospheric conditions. The standard deviations of the velocity fluctuations agree well and correlation coefficients between sounder and direct estimates were found to be in the range 0.7–0.9. This technique may offer a quantitative remote method of assessing the mixing quality of the lower atmosphere and could prove of great use in studies of atmospheric diffusion and environmental pollution.

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## Hercynian high pressure/low metamorphism temperature in the Ile de Groix Blueschists

We present here an interpretation of K–Ar ages obtained on four glaucophanes, four phengites and one barroisite separated from aluminous, calcareous and basic schists cropping out on the Ile de Groix, off southern Brittany in France. The data include two glaucophane–phengite pairs (PM6 and PC10) and a composite separate specially enriched in barroisite. (Table 1).

Field relations and chemical mineralogy are best known for the glaucophanic eclogite PC10, which occurs in boudins having axes of elongation parallel with the dominant N–S mineral lineation in the south-east of the island. This metabasic boudin lies in a matrix of chloritoid micaschist, and is apparently not an exotic block (the encasing schist has an approximately cofacial paragenesis).

The pressure/temperature conditions during the metamorphism of the PC10 glaucophanic eclogite have been inferred from the Fe/Mg partitioning between adjacent garnet and clinopyroxene rims and from the jadeite content of omphacite in equilibrium with quartz and albite<sup>3</sup> (for analysis of all minerals and rocks except PC10 see refs 1 and 2). This gives an intersection of equilibria at 400 °C and 8 K bar pressure, which is consistent with blueschist facies rocks from New Caledonia and Japan, and suggests that metamorphism took place in a region of low geothermal gradient (about 17 °C km<sup>-1</sup> if average density = 2.9 g cm<sup>-3</sup>)—possibly in a subduction zone.

The concordance of K–Ar dates for two glaucophane–phengite pairs from widely separated aluminous and basic schist outcrops on Groix demands either a simple cooling age or mineral growth age interpretation; there is no evidence in either case for excess <sup>40</sup>Ar.

If we assume a maximum prograde growth temperature of 400 °C for the Groix phengites and glaucophanes in equilibrium, it is very likely that they grew at or beneath their blocking temperatures for Ar diffusion<sup>1</sup>. Thus, the age recorded would be the peak of high pressure/low temperature metamorphism rather than the cooling age. Even if mineral reactions ceased before the system became closed to Ar diffusion, it seems unlikely, in the context of a downgoing lithospheric slab, that high pressure/low temperature conditions could prevail for any significant interval of time after the peak of metamorphism was reached<sup>5</sup>. So, because of dynamic instability of the geo-

Table 1 Mineral assemblages of whole-rock specimens\*

|       |                                                                                                                                                     |
|-------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| PM6:  | Phengite + ferroglaucophane + chloritoid + quartz + sphene + chlorite.<br>Aluminous schist, possibly metatuffaceous sediment, from Pen Men.         |
| LM8:  | Phengite + quartz + garnet + chlorite + glaucophane.<br>Aluminous schist. Common pelite lithology from Loc Maria.                                   |
| LH11: | Epidote + glaucophane + quartz + garnet + phengite.<br>'Calcareous' schist, probably from margin of metabasic body, near Loc Maria.                 |
| LH13: | Epidote + garnet + quartz + glaucophane + phengite.<br>'Calcareous' schist, probably basic metatuff from margin of metabasic body near Loc Maria.   |
| PC10: | Garnet + glaucophane/barroisite + omphacite + epidote + phengite + albite + quartz + sphene.<br>Basic schist from core of boudin, Pointe des Chats. |

\* Listed in order of decreasing modal abundance; dated mineral separates underlined.



Table 2 Data from minerals\*

| Sample no. | Mineral                         | $^{40}\text{Ar}$ rad mol g $^{-1}$<br>( $\times 10^{-8}$ ) | $^{40}\text{Ar}$ rad<br>(%) | K $_2$ O<br>(%)        | $^{40}\text{Ar}/^{36}\text{Ar}$ | $^{40}\text{K}/^{36}\text{Ar}$<br>( $\times 10^3$ ) | Age (Myr)           |         |
|------------|---------------------------------|------------------------------------------------------------|-----------------------------|------------------------|---------------------------------|-----------------------------------------------------|---------------------|---------|
| LM-8       | Phengite                        | 0.523                                                      | 95.4                        | 9.88( $\pm 0.04$ )     | 7,234                           | 329                                                 | 328.3( $\pm 9.0$ )  | } 330.2 |
| LM-8       | Phengite                        | 0.518                                                      | 87.3                        | As above               | 2,470                           | 105                                                 | 326.0( $\pm 8.8$ )  |         |
| LM-8       | Phengite                        | 0.537                                                      | 97.5                        | As above               | 11,863                          | 544                                                 | 336.3( $\pm 9.2$ )  | } 327.5 |
| PM-6       | Phengite                        | 0.544                                                      | 84.7                        | 10.28( $\pm 0.16$ )    | 1,976                           | 80.4                                                | 327.7( $\pm 10.3$ ) |         |
| PM-6       | Phengite                        | 0.543                                                      | 95.1                        | As above               | 6,815                           | 311                                                 | 327.2( $\pm 10.3$ ) | } 336.7 |
| LH-13      | Phengite                        | 0.519                                                      | 96.2                        | 9.59( $\pm 0.19$ )     | 4,851                           | 215                                                 | 334.6( $\pm 11.4$ ) |         |
| LH-13      | Phengite                        | 0.523                                                      | 83.1                        | As above               | 1,859                           | 71.9                                                | 338.9( $\pm 11.4$ ) | } 339.1 |
| PC-10      | Phengite (< 100 mesh)           | 0.577                                                      | 86.3                        | 10.64 ( $\pm 0.09$ )   | 2,230                           | 88.9                                                | 339.3( $\pm 9.7$ )  |         |
| PC-10      | Phengite (> 100 mesh)           | 0.527                                                      | 86.8                        | 9.49( $\pm 0.09$ )     | 2,311                           | 92.3                                                | 342.7( $\pm 9.9$ )  | } 317.0 |
| PC-10      | Phengite (> 100 mesh)           | 0.515                                                      | 95.3                        | As above               | 6,451                           | 288                                                 | 335.5( $\pm 9.6$ )  |         |
| PC-10      | Glaucophane (80% pure)†         | 0.00422                                                    | 34.2                        | 0.0837( $\pm 0.0010$ ) | 461                             | 8.31                                                | 313.2( $\pm 10.0$ ) | } 317.0 |
| PC-10      | Glaucophane (80% pure)          | 0.00437                                                    | 58.3                        | As above               | 765                             | 23.0                                                | 320.7( $\pm 10.2$ ) |         |
| PC-10      | Glaucophane (70% pure)†         | 0.00593                                                    | 17.8                        | 0.118( $\pm 0.002$ )   | 361                             | 3.23                                                | 313.2( $\pm 12.7$ ) | } 317.0 |
| PC-10      | Omphacite + garnet + barroisite | 0.00586                                                    | 46.3                        | 0.124( $\pm 0.001$ )   | 576                             | 15.1                                                | 294.6( $\pm 8.2$ )  |         |
| PM-6       | Ferro-glaucophane               | 0.000801                                                   | 9.2                         | 0.0151( $\pm 0.0005$ ) | 325                             | 1.40                                                | 329 ( $\pm 33$ )†   |         |
| LH-11      | Glaucophane                     | 0.000546                                                   | 6.0                         | 0.0107( $\pm 0.0005$ ) | 315                             | 1.05                                                | 318 ( $\pm 32$ )†   |         |

\* $\lambda_e = 0.585 \times 10^{-10} \text{ yr}^{-1}$ ;  $\lambda_g = 4.72 \times 10^{-10} \text{ yr}^{-1}$ ;  $^{40}\text{K}/\text{K} = 1.19 \times 10^{-4} \text{ atom/atom}$ .

†The 1 $\sigma$  precision of the age determinations for PM-6 glaucophane and LH-11 glaucophane (with  $^{40}\text{Ar}$  rad < 10%) has been evaluated at 10%.

‡Barroisite rims are the main impurity. Microprobe gives barroisite rims K $_2$ O = 0.24%, glaucophane core K $_2$ O = 0.04%.

$^{40}\text{Ar}$  was determined by isotope dilution mass spectrometry at the University of Naples, using a GD-150 Varian Mat mass spectrometer and the  $^{38}\text{Ar}$ -enriched Zurich spike. The calibration was checked by LP-6 USGS standard, a muscovite internal standard (MCT) and Bern LP-6 biotite. K was determined in duplicate by flame atomic absorption with a Perkin Elmer 306 by the method of standard additions. Errors are maximum deviations from the mean. The errors (1 $\sigma$ ) for K–Ar dates have been evaluated using the Cox and Dalrymple formula<sup>9</sup>.

thermal gradient in an area of blueschist formation, the minerals grew quickly and were uplifted soon afterwards. Thus, the growth age of minerals from such an environment would be indistinguishable within error from the cooling age, given reasonable estimates of uplift rate (see Table 2).

A high pressure/low temperature metamorphic event  $335 \pm 20$  Myr ago on Groix would postdate the intrusion of anatectic granites on the mainland by about 40 Myr (ref. 6), but would coincide with the intrusion of early Variscan granites throughout Brittany 340 Myr BP (ref. 7). This juxtaposition of blueschist with a region of active plutonism from 380 to 340 Myr BP is similar to that observed in the Mesozoic and Tertiary paired metamorphic belts of Japan and California. In the case of Groix, however, no 'median' tectonic line or suture is exposed, the blueschist 'belt' is restricted to one island, and the level of erosion of the 'hot belt' is so deep that the volcanic arc has been stripped off, revealing the underlying batholiths and migmatites.

As soon as subduction of the blueschists had ended, uplift was probably brought about by isostatic response to rapid erosion of the volcanic arc and cordillera to the north. Thermal relaxation to a more normal geothermal gradient after subduction would have produced a rise in temperature during off-loading, and a consequent retrogression of the blueschist facies minerals. If the uplift rate were fast enough, however, the blueschists of Groix would have reached the Earth's surface before the thermal relaxation 'wave' could erase much of the blueschist assemblage.

The barroisites seem to have grown at the expense of glaucophane, omphacite and garnet during a late rise in temperature that did not exceed the Ar-blocking temperature of glaucophane—otherwise the glaucophane ages would all be reset at 294 Myr BP. This would account for the fact that the barroisite overgrowths on glaucophane are significantly younger by about 40 Myr than the high pressure/low temperature metamorphic assemblage. Furthermore, the apparent slight discordance between the ages of PC10 phengite and glaucophane can be explained by the influence of younger, K-rich barroisite rims in the impure PC10 glaucophane separates. The age of this late retrogression (294 Myr BP) corresponds well with uplift and cooling ages throughout the Hercynides, and is synchronous with the intrusion of younger Variscan granites<sup>7</sup>, a major break in sedimentation and the main phase of Hercynian folding (Sudetic phase: Westphalian in age).

The Hercynian age proposed for the blueschist metamorphism on Groix is at variance with previous structural interpretations

that have related early isoclinal fold phases to the Cadomian Orogeny (690–570 Myr BP)<sup>8</sup>. The basic glaucophane schist assemblages have been considered to have developed from pre-existing, possibly Cadomian, eclogites<sup>1</sup>. By contrast, we consider it very likely that both fold structures and metamorphic assemblages on Groix are Hercynian in age, and that the original lavas and sediments need be no older than 400–500 Myr (unpublished Sr isotope data, M. S. N. C. and P. N. Taylor).

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## 'Space' charging of insulators

THE charging of satellite surfaces by space radiation often results in electrical discharges. These can interfere seriously with electrical systems on board<sup>1</sup>. The surface potentials may vary according to the differing charging properties of dissimilar materials, local changes in capacitance or differences in the radiation environment. Investigations of radiation-induced electron emission have been directed predominantly at metallic or semiconducting materials, and there is a paucity of data on the charging of dielectrics. The work reported here considers

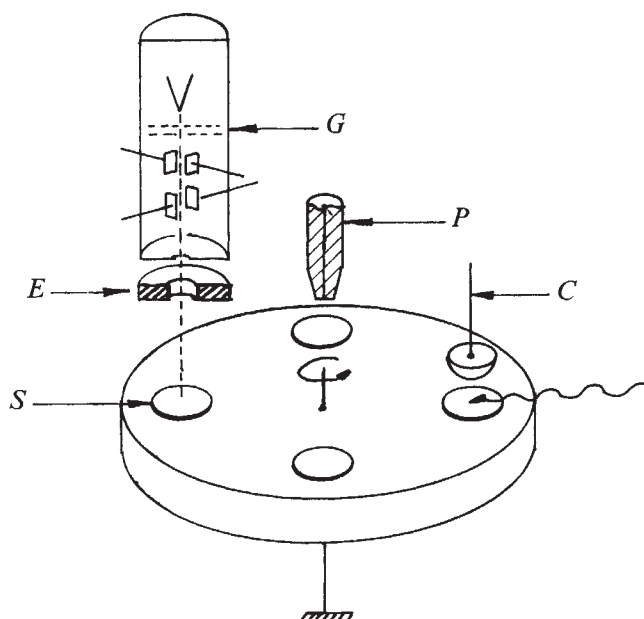


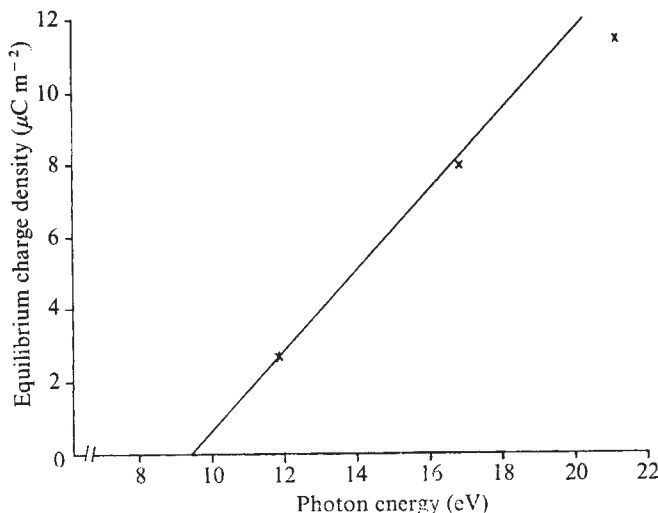
Fig. 1 Schematic diagram of experiment showing the specimen, S, mounted on the earthed, rotatable table; the counter electrode, C; an electron gun, G, with associated electrode, E; and the charge measuring probe, P.

photon-induced charging. Electron-induced charging is considered in ref. 2.

Photoelectric emission from fused silica on irradiation with ultraviolet light has been examined *in vacuo*  $\sim 10^{-5}$  mmHg. A schematic diagram of the experiment is shown in Fig. 1. Resonance radiation from, successively, a helium (21.2 eV), neon (16.85 eV) and argon (11.83 eV), discharge is directed, through a monochromator, obliquely on to dielectric specimens mounted on an earthed, rotatable table. The specimens are irradiated when positioned beneath the counter electrode, the charge being subsequently determined by scanning the specimens beneath a probe (see ref. 3).

The equilibrium charge densities produced on specimens of  $\text{SiO}_2$ , 300  $\mu\text{m}$  thick, have been measured for several extracting (positive) counter electrode potentials. Figure 2 shows the charge densities determined for zero extracting potential plotted against the incident photon energy. The line shown indicates the geometric capacitance of the specimens. The

Fig. 2 Equilibrium charge density measured on the  $\text{SiO}_2$  specimens plotted against the incident photon energy. The line shows the specimen capacitance.



saturation charge density would be expected to follow the photon energy linearly, the retarding surface potential at equilibrium being just sufficient to overcome the photoelectron emission energy, thus inhibiting emission. As evidenced, there is a tendency for the higher charge densities to be less than those predicted, and this may indicate a process of photoconductive loss.

Positive potentials applied to the counter electrode increase the equilibrium charge density (Fig. 3). Data for all of the wavelengths used are presented, again with the line showing the specimen capacitance. Evidently, the equilibrium charge density is well described by

$$\sigma = \epsilon \epsilon_0 (h\nu - \phi_p + V_a) / d, \text{ C m}^{-2}$$

where,  $\epsilon$ ,  $d$  and  $\phi_p$ , are the specimen permittivity, thickness and photoelectric threshold energy, respectively;  $h\nu$  is the incident photon energy, and  $V_a$  is the counter electrode potential.

The temporal growth of charge has been examined, the emission current being found to decrease monotonically as the surface retarding potential increases. The mode of current decay with an applied extracting field seems different from that for zero potential, suggesting that penetration of the field into the dielectric may affect the excitation process as well as increase the probability of escape of the photoelectron. These investigations will be described more fully elsewhere.

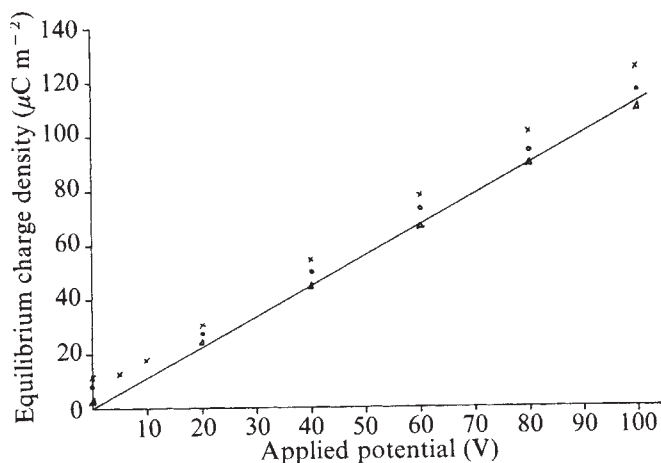


Fig. 3 Equilibrium charge density against applied counter electrode potential, for 21.2 eV (x), 16.9 eV (o) and 11.83 eV (Δ) incident radiation. Specimen capacitance shown by solid line.

These experiments have shown that photocharging by solar radiation, which is often discounted<sup>4</sup>, may not after all be insignificant, particularly in view of the extensive range of photon energies observed in the solar spectrum<sup>5</sup>. Also, it is evident that the equilibrium charges on insulators in space may be highly influenced by local electric fields.

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## Charges and vacuum flashover

THE increasing importance of vacuum insulation in technology<sup>1</sup> and the recognition of the disruptive effects of surface discharges on satellites in space<sup>2</sup> both impart fresh impetus to the understanding of the flashover effect. Solid insulators are certainly a source of gas *in vacuo*, particularly under bombardment, and can also cause local enhancement of electric stress through the acquisition of surface charges.

de Tourreil and Srivastava<sup>3</sup>, among others, have measured positive charges on insulators at stresses well below those necessary for flashover. They proposed a charging process based on secondary electron emission following bombardment of the dielectric by electrons initially field-emitted from the cathode. A secondary emission coefficient,  $\delta$ , greater than unity (more out than in) obtains for most dielectrics<sup>4</sup> but strictly between well defined limits of input electron energies, that is,  $\sim 1 \text{ eV} < E < \text{a few keV}$ , for instance. Outside these limits the insulator would charge negatively. Moreover, the emission energy of the secondary electrons is quite small,  $\sim \text{few eV}$  (ref. 5), and so very little positive charge on the insulator would preclude further emission. These effects are clearly illustrated by the simple experiment, shown schematically in Fig. 1 of ref. 6, in which electrons, with energies of up to 3 keV, are directed through a counter electrode on to insulator specimens, and the charges produced measured.

Specimens of fused silica (300  $\mu\text{m}$  thick) and of two polymers—polycarbonate (55  $\mu\text{m}$ ) and polyimide (50  $\mu\text{m}$ )—have been investigated. Figure 1 presents the measured charge densities for zero extracting field plotted as a function of the input beam energy. The upper secondary emission threshold energy ( $E_2$ ) of the primary electrons is clearly displayed. For higher input energies, the insulator charges negatively, thereby slowing incoming electrons which thus arrive at the surface with precisely the threshold energy, so that equilibrium is attained ( $\delta=1$ ). The full lines agree with the calculated geometric capacitance of the specimens to within 5%.

The occurrence of this dynamic process, rather than of complete repulsion of a high energy, primary beam (the

other stable condition) is important since it indicates continued interaction even in the highly charged equilibrium state. Local discharges would probably dissipate larger areas of charge, for example, because of the increased surface conductivity.

For input energies below the threshold ( $E_2$ ) very little (virtually random) charge is detected on the dielectrics. If an extracting field is generated, however, by applying positive potentials to the counter electrode, positive charge densities are observed, these being independent of the primary beam energy but linearly dependent on the extracting potential. Above the threshold the extracting potential has no effect.

These observations suggest that the straightforward secondary emission theory of insulator charging, leading subsequently to breakdown, may be inadequate. The electric stress at flashover is usually greater than around  $5 \text{ MV m}^{-1}$ , so the maximum free path for primary electrons in this field, if they are to collide with the insulator surface with energy less than the upper threshold energy,  $\sim 1 \text{ keV}$ , is less than 0.2 mm. Thus, for insulators of greater length, such primary electrons must originate from adjacent sites on the dielectric surface itself. That may be possible; but what happens to the electrons with greater than the threshold energy, that is, those originating further than 0.2 mm from the point of collision? They would charge the insulator negatively, irrespective of any apparent extracting field, except, perhaps, in three possible conditions:

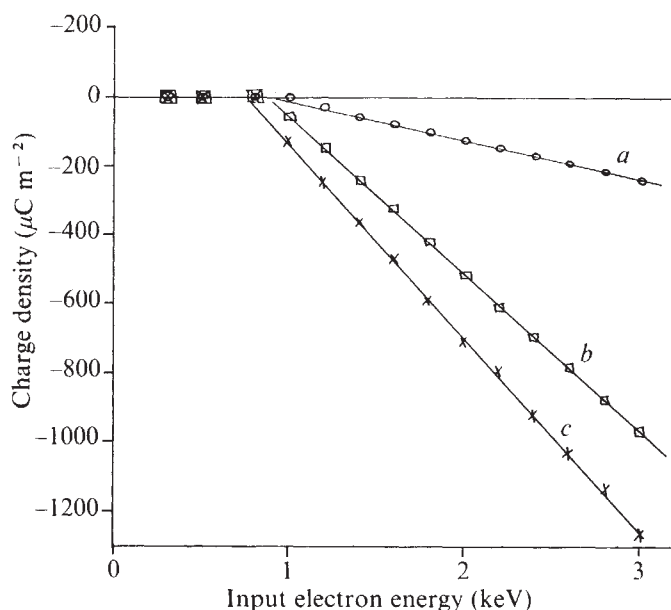
- At low (near grazing) angles of incidence the considerably reduced normal depth of penetration of the primary beam may result in the emission of secondaries, even at high primary beam energies, that is, the secondary threshold energy may be greatly increased.
- The enhanced conductivity of the irradiated surface layers of the insulator may have an important role in establishing local potential gradients.
- The collision of particles originating from both the electrode and the dielectric surfaces may have an important independent role in charging the insulator surface.

To my knowledge these aspects have not been investigated.

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Fig. 1 Charge densities measured on  $\text{SiO}_2$  (a), polycarbonate, (b), and polyimide (c), specimens, plotted against input electron energy.



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## Aspartic acid racemisation in dentine as a measure of ageing

THE L-amino acids initially present in bone protein undergo slow racemisation over geological time at a rate which is proportional to temperature<sup>1,2</sup>. We have shown<sup>3</sup> that at the human body temperature of  $\sim 37^\circ\text{C}$  aspartyl residues in tooth enamel protein also undergo racemisation at a rate which corresponds to an enrichment in the D-aspartic acid content of  $\sim 0.1\%$  per year. No D-aspartic acid increase was detected in haemoglobin, a protein with a more rapid turnover. We concluded that D-aspartyl residues accumulate in the metabolically stable protein in tooth enamel during the human lifetime as a result of *in situ* racemisation. We proposed that the irreversible first-order rate equation calculated from the enamel results could be used to deduce the age of any stable



protein from a long lived mammal and thus the age of the organism itself. The error among samples from old individuals was large, however, probably because attrition and caries reduce the amount of uncontaminated enamel, and thus tend to limit the usefulness of tooth enamel for age determinations. In this report, we show that tooth dentine is a more suitable material, and the extent of aspartic acid racemisation in this fraction can be used as a reliable indicator of mammalian age.

Measurements of the D/L aspartic acid ratio were carried out on 20 samples of dentinal protein extracted from human teeth whose donors ranged in age from 11 to 75 yr. Teeth were obtained from dentists and cleaned of any soft tissue. The teeth were desiccated, the roots removed, and the crowns ground with a mortar and pestle. Dentine fragments were separated by hand under ultraviolet light, because they fluoresce and enamel does not. The dentine fragments were cleaned by ultrasonication in double-distilled water and dilute HCl. They were then dissolved in 6 N HCl, sealed in tubes, and hydrolysed at 100 °C. Hydrolysis was for 6 h rather than 24 h, in order to minimise the amount of acid-catalysed racemisation. Since aspartic acid is released preferentially<sup>4</sup> and the enantiomers should be equally labile, total hydrolysis of the dentine protein was not necessary. The hydrolysate was dried in a rotary evaporator and desalted on Dowex 50W-X8 cation exchange resin, and the fraction containing aspartic acid isolated and derivatised as described elsewhere<sup>5</sup>.

A plot of the dentine data is shown in Fig. 1. The least squares fit of the points can be expressed by the equation

$$\ln[1 + (D/L)] = 7.87(\pm 0.39) \times 10^{-4} \text{yr}^{-1} t_d + 0.014 \quad (1)$$

where  $t_d$  is the age of the dentine in years. The correlation coefficient,  $r = 0.979$ , indicates that 96% of the variation in the D/L ratio is accounted for by the age of the tooth. The standard deviation for the dentine results is  $\pm 0.0034$ . The close agreement between the two 35-yr-old teeth, extracted from two different individuals, illustrates the reproducibility of the analyses.

The first-order rate constant  $k_{asp}$  for aspartic acid racemisation for dentine (the slope of the line in Fig. 1) falls within one standard deviation of the value for enamel. This indicates that the bulk of dentinal protein is not turned over, although the odontoblasts lining the pulp cavity remain viable throughout life and may deposit secondary dentine as a response to heavy wear<sup>6</sup>. The primary protein in enamel is non-collagenous, but 90% of the organic matrix in dentine is composed of collagen,

and there is a phosphoprotein component which may account for 20% of the aspartic acid content of human dentine. Thus, we probably have three examples of metabolically inert polypeptides in which the aspartyl residues show increasing racemisation with increasing age. Moreover, collagen comprises 25–30% of the protein in the human body, and it is distributed as connective tissue throughout every organ system. This study shows that ~6% of the L-aspartyl residues in metabolically stable protein will have racemised to the D-enantiomers by 60 yr of age. It seems reasonable to assume that some of the physicochemical properties of the molecules will be altered as a result of this small amount of racemisation, and the effects should become more significant with increasing age. If a similar racemisation process occurs in all metabolically inactive proteins, then it may be part of the complex ageing changes which take place in mammals.

These results also indicate that dentine can be used, in the same manner as enamel, for estimating the age of a mammalian tooth. In many respects, racemisation analyses of dentine should be more reliable. First, dentine accounts for a much greater proportion by weight of a tooth than does enamel. In addition, the percentage composition of protein in dentine is 100 times that in enamel<sup>8</sup>. Thus, the chances of contaminating a sample are significantly reduced when dentine is analysed. Enamel is subject to surface alterations (attrition, bacteria, caries) which could affect its amino acid composition, whereas dentine is relatively sheltered by the enamel layer. Among people experiencing heavy tooth attrition, to the extent that very little of the crown remains after 40–50 yr, root dentine could be analysed.

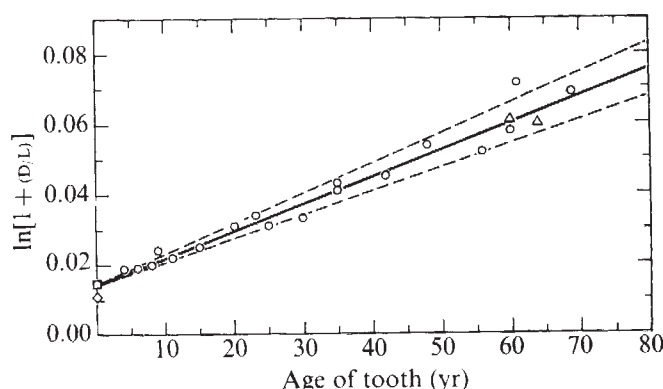
The analytical advantages of dentine over enamel are reflected by the correlation coefficients. Since the total error is  $(1 - r^2)100$ , all sources of error (experimental and contamination) amount to 4% in the dentine results, as compared with 15% for enamel. The standard deviation of the enamel values is more than twice that of the dentine values.

It is interesting that the enamel samples older than 50 yr had a standard deviation 2.4 times that of younger samples<sup>3</sup>. Although there are only four dentine samples of known age above 50 yr, a similar age-related increase in the scatter occurs with the dentine values. The standard deviation for dentine analyses of teeth greater than 50 yr old is 0.0060, compared with 0.0021 for teeth less than that age. Temperature is one factor which could cause small differences in the rate of racemisation between individuals. Human body temperature averages 36.9 °C and has a range of  $\pm 0.7$  °C (ref. 9). This temperature variation would result in a change of about  $\pm 12\%$  in the value of  $k_{asp}$  (an activation energy of 33 kcal mol<sup>-1</sup> was used in this calculation<sup>10</sup>). The broken lines in Fig. 1 represent the estimated  $k_{asp}$  values at 37.6 °C and at 36.2 °C. It can be seen that changes in the rate constant produce greater deviations among older tooth samples than among younger ones. Two standard deviations ( $\pm 2\sigma$ ) of the experimental  $k_{asp}$  for dentine fall within the range of the  $k_{asp}$  values calculated for a  $\pm 0.7$  °C difference in body temperature. Thus, the 2–3 times increase in the standard deviation among dentine and enamel samples greater than 50 yr old could be due to differences in body temperature among the individuals.

The racemisation ageing method can now be used to test claims of unusual longevity among the populations of Georgia in the USSR, Hunza, and Vilcabamba, Ecuador. Given a tooth suitable for analysis, an age estimate will fall within  $\pm 10\%$  of the individual's actual age (approximately  $\pm 2\sigma$  of the dentine distribution), and body temperature information will further reduce the error. We would appreciate the cooperation of gerontologists and dentists in obtaining tooth samples from these or other very old people.

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**Fig. 1** Plot of  $\ln[1 + (D/L)]$  for aspartic acid against age of the dentine in years (solid line). Actual ages of tooth donors are corrected, depending on the type of tooth, by the values listed in ref. 3. The slope ( $7.87 \times 10^{-4} \text{yr}^{-1}$ ) of the dentine line is equal to  $k_{asp}$  for tooth dentine at approximately 37 °C. The broken lines represent the  $k_{asp}$  values calculated for a  $\pm 0.7$  °C difference in body temperature. ○, Individual of known age; △, exact ages unknown but estimated by dentists (these points were not used to calculate the least squares fit); ◇, albumin; □, bovine tendon collagen.



PATRICIA MASTERS HELFMAN

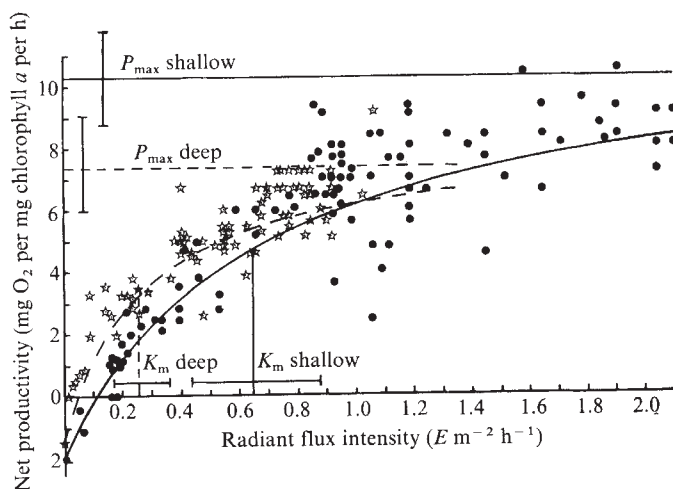
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**Fig. 1** Photosynthesis–radiation intensity relationships for *Pavona* from 10 m (●) and 25 m (open star).  $E$ , einstein ( $= 6.02 \times 10^{23}$  quanta). Ideal curves, maximum photosynthetic rates ( $P_{\max}$ ), radiant flux intensities at half saturation ( $K_m$ ), and their 99% confidence limits are from maximum-likelihood regression analysis<sup>14,15</sup>. 25-m data are from one of the two replicates. Values at zero radiant flux are means: shallow specimen  $x = -1.84$ , s.d. = 0.29,  $N = 71$ ; deep specimen  $x = -1.41$ , s.d. = 0.27,  $N = 117$ . Experiments were run for 26 h *in situ* in Perspex chambers which were stirred continuously and flushed with ambient water at 35-min intervals. The instrument design was modified from that of McCloskey<sup>22</sup>. Oxygen concentrations in the chambers were monitored with silver–platinum electrodes<sup>23</sup> and photosynthetically active radiation (400–700 nm) at the depth of the respirometers was measured with a quantum sensor (Lambda Instruments). These values were recorded continuously on strip chart recorders (Esterline Angus Minigraph). The electrodes were calibrated in air-saturated seawater and a yeast suspension in dilute sucrose solution, according to the tables of Green and Carritt<sup>24</sup>, and checked with an independently calibrated commercial oxygen meter (Yellow Springs Model 57). The *in situ* light sensor was calibrated with a factory-calibrated quantum radiometer (Lambda Instruments). After termination of the experiments, the corals were frozen. Chlorophyll was extracted in 90% acetone (20 h, dark, 5 °C). Extinctions were measured in 10-mm cells (Beckman Model DU Spectrophotometer), and total chlorophyll  $a$  was estimated by the trichromatic equations of Richards<sup>25</sup>.

## Sun and shade differences in productivity of reef corals

REEF CORALS are mutualistic symbiotic associations between cnidarians and dinoflagellates. The algae photosynthesize and translocate photosynthate to the animal partner, although the animal is capable of heterotrophic nutrition<sup>1–5</sup>. Some reef corals have wide bathymetric ranges<sup>6,7</sup> and the growth and survival of individuals in the deeper regions of the euphotic zone could result from an increase in the energy efficiency of the symbiotic complex. A number of reports document the laboratory-determined photosynthesis–radiation responses of corals<sup>8–12</sup> and zooxanthellae<sup>13</sup>, but because the details of the habitat or depth from which the animals were collected are rarely described, comparisons among results in the literature are difficult. Here we provide evidence for the existence of ‘sun’ (heliophytic) and ‘shade’ (sciophytic) differences in the net photosynthesis–radiant flux intensity response of individuals of one coral species from 10 and 25 m depth on the same reef in an atoll lagoon.

Individuals of the foliaceous coral *Pavona praelata* Dana were collected underwater at the Marine Pier Pinnacle Reef 300 m west of Enewetak Island (site Fred), Enewetak Atoll, Marshall Islands. Immediately after collection, the specimens were placed at the same depth in 1.5-l Perspex containers in which net rates of photosynthesis were determined (Fig. 1). Oxygen production per unit chlorophyll  $a$  was measured for 5-min intervals as a function of radiant flux intensity, and the data were fitted to the Michaelis–Menten equation by an iterative maximum likelihood solution method, which improves on an initial Lineweaver–Burk estimate<sup>14,15</sup>.

The best fit Michaelis–Menten curves for the *Pavona* specimens are plotted in Fig. 1 with 99% confidence limits (one-tailed Student's  $t$  test,  $n-2$  d.f.) for  $P_{\max}$  (maximum photosynthetic rate) and  $K_m$  (radiant flux intensity at half saturation). By analogy to enzyme kinetics,  $K_m$  is a measure of the affinity of the photosynthetic system for radiant energy. The lack of overlap of the confidence limits indicates that the  $K_m$  for the 25-m specimens are lower than the value for the 10-m specimen (Table 1). Therefore, relative to their maximum photosynthetic rates, the deep water individuals saturate their photosynthetic machinery at lower radiation intensities than the shallow water individual. This is analogous to the ‘sun’ and ‘shade’ differences documented for canopy and understory plants<sup>16</sup> and for phytoplankton acclimated to high and low light intensities<sup>17,18</sup>. The saturation of deep water individuals at lower radiation intensities is consistent with Margalef's<sup>19</sup> observation of greater  $A_{450}$  of pigments from deep water

corals compared with conspecifics from shallow water. The measured difference in photosynthetic response is not likely to be due to spectral quality changes in the light between 10 and 25 m, because the 640–670-nm light which affects the red action spectrum peak of photosynthesis<sup>13</sup> amounts to only 2% of the total light flux at 18 m and 0.03% at 25 m (ref. 20), a difference below the resolution range of our *in situ* light meter circuit. We found no difference in temperature between study sites, and von Arx<sup>21</sup> has estimated that atoll lagoons have complete water overturn every 150 h, indicating that temperature stratification is unlikely. Therefore we believe that the differences in photosynthetic response between the two study sites are not temperature related.

Franzisket's<sup>11</sup> data from specimens collected at 3 m indicate  $K_m$  values higher than our 25-m specimens and within the range of our 10-m samples, corroborating our interpretation that ‘sun’ and ‘shade’ differences exist in the photosynthetic response of corals from high and low light regimes (Table 1). Evidence in support of the phenomenon comes from our *in situ* data for *Plerogyra*, *Porites* and *Acropora* from the Philippines and from Barnes and Taylor's<sup>12</sup> *in vitro* data on *Montastrea annularis* from the Caribbean (Table 1). The 18-m depth specimens have  $K_m$  values in the range of our 25-m *Pavona*, the *Montastrea* from 15-m is intermediate between our 25-m and 10-m *Pavona*, and the *Acropora* from 3 m exhibits a  $K_m$  in the range of Franzisket's<sup>11</sup> 3-m specimens and our 10-m *Pavona*. The overlap in  $K_m$  among the different coral species



Table 1 Half-saturation constants for photosynthesis as fit to the Michaelis-Menten\* equation

| Species                                                      | Depth (m) | Maximum likelihood estimate | $K_m$ ( $E\ m^{-2}\ h^{-1}$ ), One-tailed confidence limits<br>Lower ( $P < 0.01$ ) Upper |      |
|--------------------------------------------------------------|-----------|-----------------------------|-------------------------------------------------------------------------------------------|------|
|                                                              |           |                             |                                                                                           |      |
| <i>P. praetorta</i> Dana                                     | 25        | 0.26                        | 0.18                                                                                      | 0.37 |
| <i>P. praetorta</i> Dana                                     | 25        | 0.14                        | 0.00                                                                                      | 0.38 |
| <i>P. praetorta</i> Dana                                     | 10        | 0.63                        | 0.43                                                                                      | 0.88 |
| <i>Pterogyra sinuosa</i> Dana                                | 18        | 0.18                        | 0.10                                                                                      | 0.26 |
| <i>Porites lutea</i> Milne Edwards and Haime                 | 18        | 0.11                        | 0.07                                                                                      | 0.15 |
| <i>Acropora</i> cf. <i>arbuscula</i> Dana                    | 3         | 1.23                        | 0.76                                                                                      | 1.99 |
| Kaneohe Bay corals, Hawaii† (ref. 11)                        | 3         | 0.65                        | 0.46                                                                                      | 0.86 |
| <i>Montastrea annularis</i> Ellis and Solander <sup>12</sup> | 15        | 0.41                        | 0.21                                                                                      | 0.67 |

Each line represents a 24-h incubation of a single coral.

\*Michaelis-Menten equation:  $P = (P_{\max} \times I)/(K_m + I)$  or  $P_{\text{relative}} = P_{\text{observed}}/P_{\max} = I/(K_m + I)$

†Pooled data from four species: (*Fungia scutaria* Lamarck, *Montipora verrucosa* Lamarck, *Porites compressa* Dana and *Pocillopora meandrina* Verrill); regressions run separately were not significantly different ( $P > 0.05$ ).

suggests that, given a particular light regime, the algal photosynthetic efficiency relative to the maximum photosynthetic rate is independent of the coral species that the algae inhabit. In addition, Kawaguti<sup>8</sup> found the compensation light intensity was related to habitat: individuals from deeper or shaded places showed lower values.

The existence of acclimation to ambient light regime in corals suggests that extrapolation from measurements on individuals at one depth to the behaviour of the population over the whole depth range is not valid. Similarly, the efficiency of translocation of photosynthate from alga to animal partner may vary with depth. *In situ* 24-h measurements on individuals from a range of depths and species are needed to begin to resolve the question of the trophic plasticity of corals as a function of habitat.

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## Frost sensitivity of *Zea mays* increased by application of *Pseudomonas syringae*

DRIED, powdered corn leaves (*Zea mays* L.) dusted on to the leaves of corn seedlings, will increase frost damage to treated seedlings relative to untreated controls at  $\sim -4^\circ\text{C}$  (refs 1, 2). In a preliminary attempt to isolate the entity responsible for this activity, we found that water extracts of corn leaf powder from healthy field-grown plants caused as much increase in frost sensitivity when applied as a spray to seedling corn, as did application of the powder itself when tested as described below. Addition of either streptomycin or tetracycline (1,000 p.p.m.) to these water extracts eliminated their activity, suggesting the possible role of bacteria in this effect.

Corn seedlings for frost sensitivity measurements were grown in sterilised vermiculite in a plant growth chamber (day: 18 h,  $30^\circ\text{C}$ ; night: 6 h,  $20^\circ\text{C}$ ) to the 3-leaf stage. Plants were watered daily with nutrient solution. Ten or more pots (6 plants per 10-cm pot) were used for each treatment. Unless otherwise noted, treatment was given 24 h before freezing and the plants were held in a mist chamber until 20 min before freezing. Immediately before freezing, all pots were positioned randomly in a controlled temperature chamber at  $\sim 0^\circ\text{C}$ . The air temperature was lowered at  $\sim 0.1^\circ\text{C min}^{-1}$  to  $-3.0^\circ\text{C}$  and then at  $\sim 0.04^\circ\text{C min}^{-1}$  to  $-3.5$ – $-4.0^\circ\text{C}$ . Maximal differences in frost damage between treated and untreated plants occurred in this temperature range. The air temperature in the chamber was then increased at  $\sim 1.0^\circ\text{C min}^{-1}$  to  $30^\circ\text{C}$ . Frost damage was assessed by counting the number of leaves per plant with symptoms of frost injury.

Forty-two bacterial isolates of varying colony colour and morphology were obtained by dilution plating from water extracts of corn leaf powder. Each isolate was grown on nutrient agar, suspended in 0.1 M phosphate buffer, pH 7.0, sprayed on to corn seedlings and assayed for the ability to increase frost sensitivity of corn at  $-3.5$  to  $-4.0^\circ\text{C}$ . Only one of the 42 isolates was active in increasing the frost sensitivity of corn seedlings. When leaves of corn seedlings were sprayed with suspensions of this isolate in buffer 24 h before freezing  $> 95\%$  of the leaves were damaged at  $-3.5$  to  $-4.0^\circ\text{C}$ , whereas  $< 8\%$  of the leaves of plants sprayed with buffer alone were damaged at this temperature. On the basis of its response to standard physiological tests<sup>3,4</sup>, this isolate was identified as *Pseudomonas syringae* van Hall, a species that includes strains pathogenic to a large number of diverse host plants including corn<sup>5</sup>. We have not observed pathological symptoms even when corn

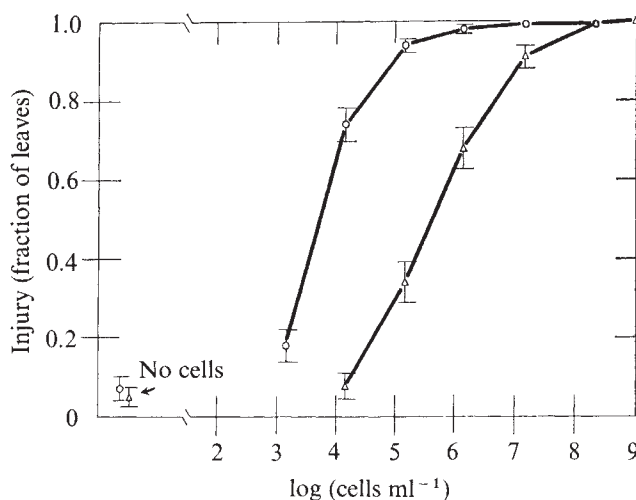


plants were held without freezing for up to 10 d after inoculation.

For ~ 8 h after application of the bacteria, the frost sensitivity of corn plants did not increase. It increased steadily from ~ 8 h until ~ 24 h (Fig. 1), after which little change occurred for at least 5 d. The amount of frost damage observed in seedling corn plants increased with increased *P. syringae* populations applied<sup>6</sup>. At each time interval shown in Fig. 1, more leaves sprayed with  $4 \times 10^6$  cells  $\text{ml}^{-1}$  were damaged than those sprayed with  $4 \times 10^5$  cells  $\text{ml}^{-1}$ . As indicated in Fig. 2, suspensions as dilute as  $1.5 \times 10^4$  cells  $\text{ml}^{-1}$  caused significant increases in frost sensitivity (75% damage for plants held under mist for 24 h before freezing). Application of higher *P. syringae* populations (~  $10^7$  cells  $\text{ml}^{-1}$  with wet leaves or  $10^8$  cells  $\text{ml}^{-1}$  with dry leaves) resulted in frost damage to nearly all leaves; untreated control plants showed damage to fewer than one leaf in 12. Plants held in a mist chamber for 24 h between application of cells and freezing, sustained greater frost damage at all applied cell densities than plants left dry during this period. The increase in frost sensitivity both with moisture and with time suggest that either growth of the bacteria or movement to some favourable site(s) on or in the leaves, or both, are important in enhancing frost sensitivity.

Sixty-four of the 67 tested isolates of *P. syringae* increased the frost sensitivity of corn seedlings. Three of five isolates of the closely related species, *P. coronafaciens*<sup>7</sup> also were active, whereas one isolate each of *P. tabaci*, *P. glycinea*, *P. phaseolicola*, *P. marginalis*, four isolates of *Erwinia stewartii* and one of *Escherichia coli* were not active.

Even plants very sensitive to frost can withstand temperatures slightly below 0 °C (refs 8–11) because of the supercooling of intracellular water. Frost damage to such plants in the field has been reported to occur at ~ -2–-5 °C, presumably by explosive crystallisation of supercooled intracellular water<sup>8</sup>. Similarly, almost all our growth chamber-grown plants with *P. syringae* on their leaves were killed at -4 °C (Fig. 2). Plants lacking leaf surface populations of *P. syringae*, however, were not substantially damaged until the temperature reached was ~ -8.0 °C (Fig. 3). Thus, our *P. syringae*-treated plants seem to be almost equal in frost sensitivity to plants in the field. It is our growth chamber-grown controls, lacking leaf popula-

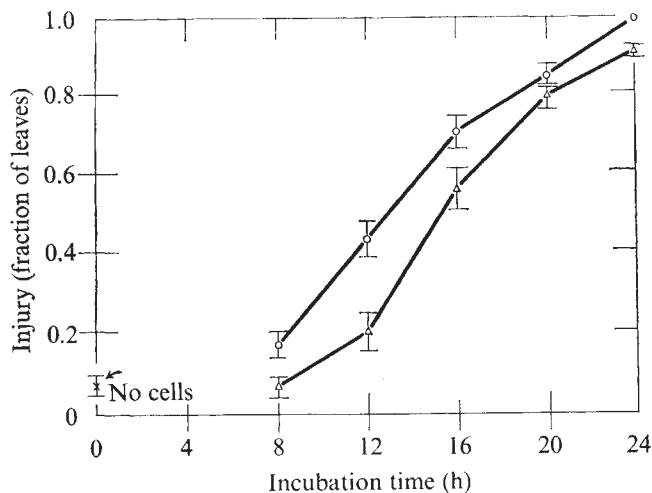


**Fig. 2** Effect of applied *P. syringae* populations on frost injury to corn seedlings. Three-leaf-stage corn seedlings were sprayed with cell suspensions in 0.1 M phosphate buffer pH 7.0 (0.5 ml per plant) and held in a dark mist chamber at 24 °C (○) or left dry in the dark at 24 °C (△) for 24 h before freezing. The vertical bars represent the s.e.m.

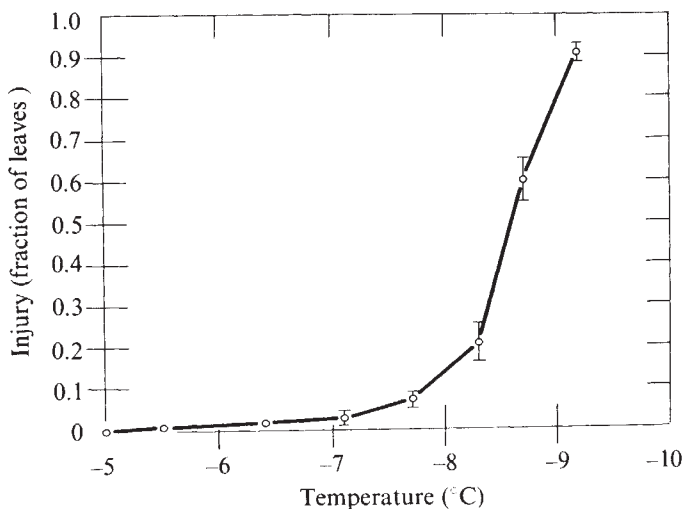
tions of *P. syringae*, that differ substantially from field-grown plants with regard to frost sensitivity.

Although the importance of ice nuclei in initiating ice crystallisation, and thus frost damage, has been recognised<sup>11</sup>, the nature and source of these ice nuclei remain unknown. Maki *et al.*<sup>12</sup> have reported that *P. syringae* is active as an ice nucleus at temperatures as warm as -2 to -3 °C. We have found that all 64 isolates of *P. syringae* and all 3 of *P. coronafaciens* which increased frost sensitivity of corn were also active in ice nucleation at -5 °C or above as measured by a modification of the procedure of Vali<sup>13</sup>. All isolates which did not increase the frost sensitivity of corn (including the 41 inactive isolates from water extracts of corn leaf powder) were not active in ice nucleation at

**Fig. 1** Effect of the duration of the time between application of *P. syringae* and freezing on frost injury. Three-leaf-stage corn seedlings were sprayed with cell suspensions of  $4 \times 10^5$  (△) or  $4 \times 10^6$  (○) cells  $\text{ml}^{-1}$  in 0.1 M phosphate buffer pH 7.0 (0.5 ml per plant) and incubated in a mist chamber for the times shown on the abscissa before freezing. The vertical bars represent the s.e.m.



**Fig. 3** Frost sensitivity of corn seedlings lacking leaf surface populations of *P. syringae*. Three-leaf-stage corn seedlings were placed in a controlled environmental chamber at 0 °C and were cooled at the rate of ~0.05 °C  $\text{min}^{-1}$ . Groups of plants were removed from the chamber at temperatures shown on the abscissa and placed in a growth chamber at 30 °C. The vertical bars represent the s.e.m.



-5 °C. Thus, the decreased frost sensitivity of our untreated control plants may be caused by supercooling to temperatures below those that are possible in the presence of active bacterial ice nuclei (for example, *P. syringae*).

The part played by epiphytic bacteria in promoting frost sensitivity has not been recognised previously. Large epiphytic bacterial populations exist on the leaves of most plants<sup>14-16</sup>. *P. syringae* has been reported as a component of these epiphytic populations on host and non-host plants<sup>16,17,19</sup>. Thus, it may be that 'natural' frost sensitivity of many plants is influenced, or even determined, by the presence of *P. syringae* and possibly certain other epiphytic bacteria.

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## Fog basking by the Namib Desert beetle, *Onymacris unguicularis*

THE Namib Desert along the south-western coast of Africa supports a sand dune fauna without counterpart elsewhere in the world<sup>1</sup>. The trophic base of the arthropod fauna is wind-blown detritus<sup>2</sup>. Aperiodic advective fog collection from vegetation<sup>3</sup> or detritus<sup>4</sup> is a possible source of water for diverse Namib animals. For the specialised fauna living in vegetation-less dunes, fog collection from detritus<sup>4</sup>, disturbed sand projections<sup>5</sup>, directly from humid air<sup>6</sup>, or from water precipitated on the body<sup>4,7</sup>, seem to be the only possible water uptake methods. Water uptake from saturated or subsaturated air, demonstrated for a few arthropod species<sup>8</sup>, is not a physiological capability of Namib tenebrionids already investigated<sup>4,6,7</sup>.

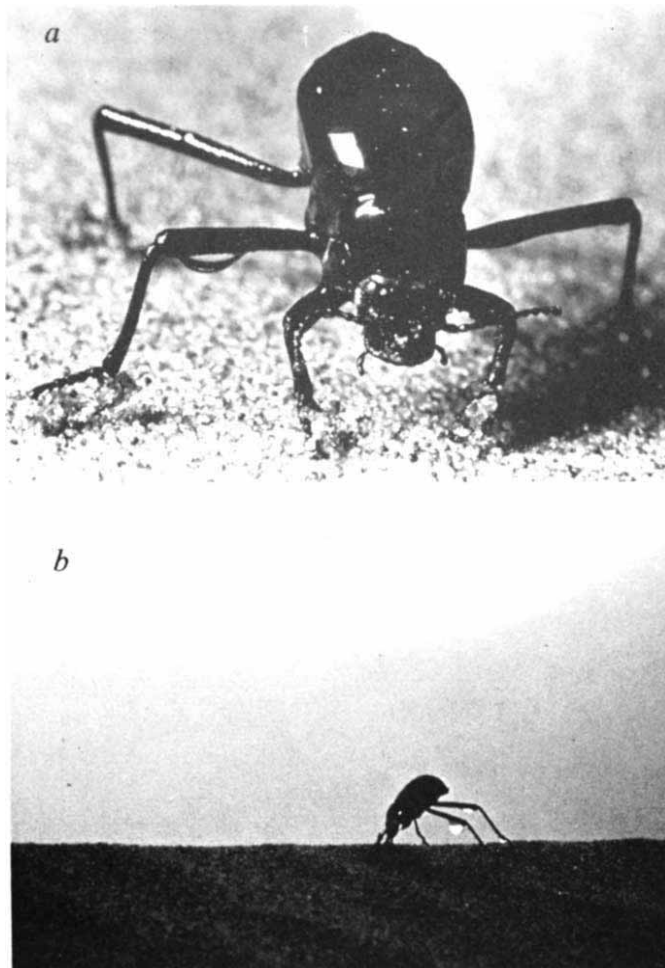
Here we report field observations of direct water uptake from fog precipitated on the body of the tenebrionid beetle, *Onymacris unguicularis*. In early October 1975 we established a study area on sand dunes south of the Kuiseb River at Gobabeb (23°34'S, 15°03'E, elevation 408 m), South West Africa, and continued observations through January 1976. On a series of small linear dunes all *O. unguicularis* were collected and marked with small (diameter 2.5 mm)

numbered disks glued to the thorax. The study area, including 5 km of dune ridges, was traversed every afternoon, during the peak activity period of these beetles. We took all beetles collected to the laboratory, weighed them, and returned them to their dunes within 2 h of capture. The beetles either burrowed into dune slipfaces when returned or remained active, feeding on detritus for an hour or more before retiring to subsurface sands. Collections were repeated daily and numbering continued until over 90% of all beetles collected were numbered individuals. Recollection of marked individuals was facilitated in this environment because, in spite of the large spatial dimension of the study area there is no vegetation and these large (13-22 mm) black beetles stand out against the reddish sand substrate.

Ordinarily *O. unguicularis* are diurnal, preferring the warm part of day, and remaining buried in the sand of dune slipfaces overnight. But during nocturnal fogs they emerge and climb the slipface to the crest. There they take a characteristic head-down stance (Fig. 1), facing into the fog-laden wind. Dune slipfaces oriented into the wind also intercept fog. Beetles emerging there adopt the typical head-standing posture where they emerge.

As water collects on these beetles it trickles down the body to the mouth. Actual uptake of water was established by collecting beetles at the end of the head-standing performance, when they burrowed into subsurface sands. They were then taken to the laboratory and allowed to dry for 2 h. All adhering sand grains were removed with a brush and the beetles were weighed. Fog water uptake ranged from an

Fig. 1 *a*, Windward view of *Onymacris unguicularis* in a typical fog-collecting stance. *b*, Another individual at the usual fog-collecting site, the crest of a sand dune. Note the water collected under the head, which is being drunk.



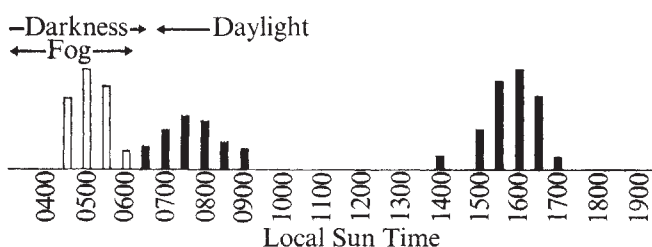
**Table 1** Fog water gain expressed as percentage weight change of total body weight following fogs of various strengths on the Namib Desert

| Date              | Fog (mm) | Mean   | Weight change (%) |         |         | N  |
|-------------------|----------|--------|-------------------|---------|---------|----|
|                   |          |        | Corrected mean    | Minimum | Maximum |    |
| October 14, 1975  | 0.50     | +13.36 | +14.50            | +3.57   | +34.01  | 24 |
| November 5, 1975  | 0.35     | +0.49  | +1.63             | -1.99   | +10.48  | 32 |
| November 25, 1975 | 0.10     | +4.33  | +5.47             | -5.76   | +16.31  | 52 |
| November 29, 1975 | 0.65     | +7.25  | +8.39             | -2.19   | +20.79  | 33 |
| December 1, 1975  | 0.10     | +0.97  | +2.11             | -2.46   | +4.91   | 16 |

The correction factor of 1.14% determined to be the mean overnight weight loss without fog has been added to the mean to provide a corrected mean weight change during fog more closely approximating the actual uptake of fog water but is not applied to the maximum and minimum weight changes.

average of 12% to no gain for various fogs (Table 1). The maximum weight gain by an individual in a single fog was 34% of total body weight before the fog. These values are for beetles captured and weighed the evening before a fog, and recaptured the morning immediately following the fog.

To determine the weight changes from evening weighing to morning weighing in the absence of an intervening fog, a control sample of beetles was weighed, marked and placed on a dune in the evening, then recaptured the following fogless morning. Of 48 such recaptures all but three individuals lost weight ( $\bar{x}$  change -1.14%, range -3.00% to +0.72%). Thus any mean weight increase by a population of beetles overnight during a fog can be assigned to uptake of fog water.



**Fig. 2** The timing of activity by *O. unguicularis* on November 12, 1975. The open columns represent individuals holding the fog-collecting posture. The dark columns are individuals actively engaged in other activities. The maximum number of active individuals at any time was 45 on this date.

Fog basking by *O. unguicularis* occurs at times when they are not ordinarily active (Fig. 2). They may emerge any time during the night that wet fogs occur. Their usual foraging periods occur during the morning and late afternoon, coinciding in the afternoon with the strongest winds which transport the detritus on which they feed.

*Onymacris unguicularis* is active throughout the year and thus encounters variable intervals between fogs. Fogs as light as 0.1 mm on the Gobabeb fog screen elicited fog basking.

Another group of Namib dune tenebrionid beetles, genus *Lepidochora*, living near Gobabeb build ridges in the sand to catch water<sup>3</sup> and we have observed spiders, lepismids and other tenebrionids active on foggy mornings. Direct collection of fog water seems to be important in the water economy of some Namib Desert animals, and to involve in certain cases highly developed methods.

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## Nucleating agents in the haemolymph of insects tolerant to freezing

MANY insects that survive the winter in cold, exposed habitats in arctic or temperate regions are sensitive to freezing. These insects usually have supercooling points below -15 °C, and their capacity for supercooling has been shown to increase considerably with increasing concentrations of glycerol in their body fluid<sup>1</sup>. On the other hand, adult insects that are tolerant to freezing ('freeze tolerant') seem to freeze invariably at high subzero temperatures (from -6 to -10 °C), without regard to the concentration of glycerol and other polyols in their body fluid<sup>2-4</sup>. We report here that haemolymph from freeze-tolerant adult beetles contains a substance, which induces freezing in the haemolymph at about -6.5 °C. Haemolymph from freeze-sensitive species seems to lack this substance. Such substances probably constitute the physical basis of the remarkably high supercooling points of freeze-tolerant adult insects.

During the winter of 1975 we found that several species of tenebrionid beetle, inhabiting the mountain areas of southern California, were tolerant to freezing. These beetles had supercooling points from -5.5 to -7.0 °C, whereas freeze-sensitive beetles froze at temperatures from -12 to -20 °C. Preliminary experiments showed that 5-μl samples of isolated haemolymph from freeze-tolerant (*Eleodes blanchardi* Blaisd.) and freeze-sensitive (*Iphthimus laevis* Csy.) tenebrionids froze within the same temperature range as did the respective intact beetles. Mixtures of haemolymph from the two species, however, also froze within the range of the intact freeze-tolerant beetles, even when the haemolymph of this species constituted as little as 5% of the volume of the mixture. These observations indicate that haemolymph from freeze-tolerant *E. blanchardi* contains nucleating agents, which induce freezing at a few degrees below zero, and which probably cause the low supercooling capacity of these beetles. Similar nucleating agents might be the cause of the high supercooling points reported for other freeze-tolerant beetles.

If freezing at high subzero temperatures is physiologically important to freeze-tolerant insects, they would be expected to contain nucleating agents, except in cases where freezing, as described by Salt<sup>5</sup>, is initiated by inoculation. To investigate the distribution of nucleating agents, we took



**Table 1** Supercooling points of intact beetles and of samples of 0.9% solution of NaCl containing haemolymph from beetles of the same species

| Species                                                | Locality and season of collection | Freeze tolerance | Supercooling points (°C) |                        |
|--------------------------------------------------------|-----------------------------------|------------------|--------------------------|------------------------|
|                                                        |                                   |                  | Intact beetles*          | 0.9% NaCl+ haemolymph† |
| <i>Eleodes blanchardi</i> Blaisd.                      | M, W                              | +                | -6.3±0.6 (n=8)           | -5.7±0.3 (n=4, m=12)   |
| <i>Eleodes blanchardi</i> Blaisd.                      | M, S                              | -                | -9.3 (n=1)               | -15.3±3.1 (n=2, m=7)   |
| <i>Eleodes omisla</i> LeC. ssp. <i>pygmaea</i> Blaisd. | C, S                              | ?                | —                        | -18.0±2.6 (n=1, m=4)   |
| <i>Coelocnemis magna</i> LeC.                          | M, W                              | +                | -6.4 (n=1)               | -6.3±0.6 (n=1, m=3)    |
| <i>Coelocnemis magna</i> LeC.                          | M, S                              | -                | -6.8 (n=1)               | -6.4±0.0 (n=1, m=4)    |
| <i>Coelocnemis magna</i> LeC.                          | C, S                              | ?                | —                        | -18.6±1.7 (n=1, m=4)   |
| <i>Coelocnemis californicus</i> Mann.                  | M, W                              | +                | -6.8±0.4 (n=2)           | -5.9±0.2 (n=1, m=3)    |
| <i>Coelocnemis californicus</i> Mann.                  | M, S                              | -                | -8.8±1.1 (n=2)           | -14.4±2.5 (n=2, m=8)   |
| <i>Iphthimus laevis</i> Csy.                           | M, W                              | -                | -15.9±1.7 (n=3)          | -17.1±2.8 (n=2, m=6)   |
| <i>Rhagium inquisitor</i> L.                           | N, W                              | -                | -17.0±2.1 (n=5)          | -18.2±5.4 (n=1, m=4)   |

Beetles of the genus *Coelocnemis* are classified according to Doyen<sup>6</sup>.

M and C, beetles collected in the mountains and on the coast in southern California, respectively; N, beetles collected in Norway; S and W, beetles collected in summer and winter, respectively.

\*Mean ± s.d. of n beetles.

†Mean ± s.d. of m parallel 5-μl samples of NaCl solution, containing about 5% of haemolymph obtained from n beetles. Samples of NaCl solution were kept inside thin glass capillaries, and the haemolymph was added by means of a microsyringe. Supercooling points were determined by cooling at a rate of 20 °C h<sup>-1</sup>, while the temperature was measured by means of a copper-constantan thermocouple. Supercooling points were indicated by a sudden increase in temperature, due to the release of heat of fusion of water.

samples of haemolymph from several species of beetles representing different kinds of water survival, and examined their effect on the supercooling points of samples of 0.9% solution of NaCl. The survey included both freeze-tolerant and freeze-sensitive species, which were collected in the mountains of southern California and around Oslo. We also included beetles collected on the coast near San Diego, where they are probably never exposed to subzero temperatures. Beetles were collected in summer and winter and kept in the laboratory at +4 °C and +20 °C, respectively, for up to 1 month. They were fed on dry oak leaves and pieces of apple, but starved for 1–2 d before being used, to prevent possible nucleators in the gut contents from influencing the supercooling points of the intact beetles. To judge whether beetles were freeze tolerant or not, we cooled them at a rate of about 20 °C h<sup>-1</sup> until they froze spontaneously. When the temperature of a freezing beetle had returned to its supercooling point, it was thawed slowly. To be classified as freeze tolerant, the beetles had to behave in an apparently normal way during the following 2 d, while kept at room temperature. The beetles here classified as freeze sensitive all looked dead after thawing, and they did not recover during the period of observation.

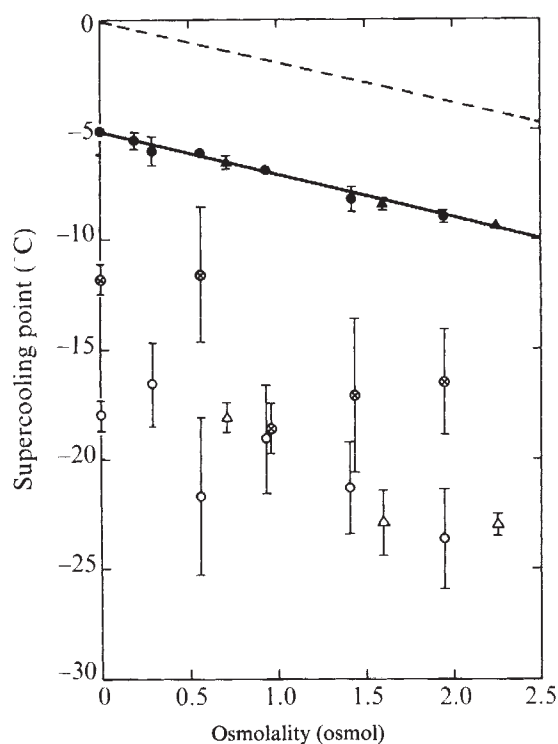
The results in Table 1 show that haemolymph from all freeze-tolerant beetles induced freezing in the NaCl solutions at temperatures of -5.4–-6.9 °C. During summer these beetles became sensitive to freezing, and at this time the freezing-inducing effect of their haemolymph was usually lost too. One exception was a freeze-sensitive *Coelocnemis magna* LeC.<sup>6</sup>, which was collected in the mountains in the summer, and which had nucleating agents in its haemolymph. No other beetles had haemolymph which altered the supercooling points of NaCl solutions significantly, indicating that the haemolymph of these species lacked nucleating agents. The coastal species were not examined with respect to freeze tolerance, but they were presumably freeze sensitive. These results suggest that only freeze-tolerant species have nucleating agents in their haemolymph, and that these agents are usually present in winter only.

Table 1 shows that summer beetles of the freeze-tolerant species, which lacked nucleating agents in their haemolymph, had supercooling points 1–3 °C lower than beetles collected in winter. This indicates the presence of other, possibly intracellular, nucleating agents, the effect of which would be masked by the nucleating agents in the haemolymph during winter.

When freeze-tolerant *E. blanchardi* were kept at +20 °C

for 2 weeks (not shown in Table 1), they became sensitive to freezing. The nucleating effect of their haemolymph, however, was still present, and the supercooling points of the intact beetles did not change, indicating that freezing at high subzero temperatures in itself does not make the beetles freeze tolerant. Furthermore, it reveals that in these

**Fig. 1** Supercooling points of solutions of NaCl (○) and glycerol (△), solutions of NaCl (●) and glycerol (▲) containing 5% of haemolymph from freeze-tolerant *Eleodes blanchardi*, and solutions of NaCl (⊗) containing 5% of the same haemolymph previously heated to +100 °C for 5 min, all plotted as a function of the osmolality of the solutions. Each point is the mean of 4–12 parallel 5-μl samples, and the bars represent the standard deviation. The regression line of points representing solutions containing native haemolymph is indicated by the solid line, and the theoretical melting points are indicated by the dashed line. The osmolality is calculated from the concentration of solute according to data from the *Handbook of Chemistry and Physics*<sup>8</sup>. The change in osmolality caused by the addition of haemolymph to the solutions is small and has not been adjusted for in the calculations.



circumstances there is a time lag between the inactivation of the nucleating agents and that of other factors which cause freeze tolerance in the beetles. A similar time lag might explain the presence of nucleating agents in the haemolymph of the freeze-sensitive *C. magna* (Table 1).

To learn more about the properties of the ice nucleators in the haemolymph, we examined the effect of haemolymph from *E. blanchardi* on the supercooling points of solutions of NaCl and glycerol. Several concentrations of solutes were used, so that the osmolality varied from near 0 to about 2 osmol. Corresponding values of osmolality and supercooling points of both pure solutions and solutions containing haemolymph are shown in Fig. 1, which shows that haemolymph from *E. blanchardi* caused a considerable increase in the supercooling points of the solutions. The supercooling points of solutions containing haemolymph are linearly related to the osmolality, and the points representing NaCl and glycerol solutions lie on the same line, indicating that the effect of glycerol is due only to its osmotic activity. The calculated regression line for all points representing solutions containing haemolymph has the formula

$$y = -5.3 - 1.95x$$

The slope of this line ( $-1.95^{\circ}\text{C}$  per osmol) is approximately the same as the osmolal freezing point depression ( $-1.86^{\circ}\text{C}$  per osmol), which is indicated by the dashed line on Fig. 1. Thus the supercooling capacity of solutions containing haemolymph does not change significantly as the osmolality of the solution changes, indicating that the nucleating agents induce freezing at a certain degree of supercooling, regardless of the osmolality of the solutions. This degree of supercooling corresponds to the difference in ordinate values of the two lines, that is  $5.3^{\circ}\text{C}$ . The dispersion of the points around the regression line is remarkably low, suggesting that when the solutions are supercooled by  $5.3^{\circ}\text{C}$ , there is a large increase in the statistical probability that an ice nucleus will form.

By heating the haemolymph to  $+100^{\circ}\text{C}$  for 5 min before it was added to the solutions, the supercooling points were lowered markedly, indicating that the nucleating agents are temperature sensitive. Some nucleating effect was still present, however, since the new supercooling points were still above those of solutions without haemolymph.

Freeze tolerance in adult insects has been known only during the past few years, and little is known about the mechanisms involved. Data from different kinds of freeze-tolerant organisms, including beetles<sup>2</sup>, indicate that ice formation is limited to the extracellular compartments, and that extracellular ice protects the organisms against intracellular freezing, which is believed to be injurious. According to this model, extracellular ice, which is considered to be unable to penetrate cell membranes, concentrates the extracellular solutes and causes an osmotic flux of water out of the cells. Thus, at sufficiently low freezing rates, allowing water to leave the cells as the freezing proceeds, the intracellular as well as the extracellular fluid would be kept at or near its melting point<sup>7</sup>. Consequently, the probability of an injurious intracellular freezing would become very small. The presence of nucleating agents in the haemolymph of freeze-tolerant beetles, ensuring extracellular freezing at high subzero temperatures, gives further support to this model.

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## Induction of male recombination in *Drosophila melanogaster* by injection of extracts of flies showing male recombination

MORGAN<sup>1,2</sup> was the first to report that no spontaneous recombination occurs in males of *Drosophila melanogaster*. Since then recombination has been induced in *D. melanogaster* males by various treatments<sup>3</sup>, for example, X rays. In an attempt to study the control of recombination in *D. melanogaster*, Reddi *et al.*<sup>4</sup> injected males with ovarian extracts and, surprisingly, induced recombination. Subsequent attempts to repeat this experiment failed<sup>5,6</sup>. It is known that some natural populations of *D. melanogaster* show spontaneous male recombination<sup>7–10</sup>, and it has been suggested that this may be caused by episomes or viruses<sup>8,9,11</sup>. It is therefore possible that these male recombination-inducing factors could be transferred by injection, and that the failure to reproduce these results<sup>4</sup> might have been due to the use of strains lacking such factors. We have therefore tested whether or not male recombination could be induced by injecting extracts of flies from strains showing male recombination into flies lacking male recombination.

An extract of *OK1* flies, a strain from Oklahoma that shows male recombination<sup>12</sup>, was prepared by homogenising together 10 each of newly emerged males and females in 4 ml of *Drosophila* Ringer solution, and centrifuging at 2,000 r.p.m. for 2 min. The supernatant was injected into virgin females which were homozygous for the recessive chromosome markers, *dp b cn bw*; *ve* (dumpy wings, 2–13.0, black body colour, 2–48.0, cinnabar eye colour, 2–57.5; brown eye colour, 2–104.5; veinlet wings, 3–0.2) until their abdomens were visibly distended. These females were mated individually to *Canton-S* (*CS*) males, a wild-type strain which when crossed to the marker strain gives *F*<sub>1</sub> males that produce no recombinants. The heterozygous male progeny were mated individually to *dp b cn bw*; *ve* females every 3–5 d for a total of four transfers. Their progeny were then scored for second chromosome male recombination. All presumptive recombinants were tested by backcrosses to *dp b cn bw*; *ve* flies. Control flies were injected with *CS* fly extract or *Drosophila* Ringer solution.

The results are summarised in Table 1. Male recombination occurred only in sons of females injected with *OK1* extract. The pattern of recombination along the second chromosome (Table 1) was similar to that found in *OK1/dp b cn bw* males<sup>12</sup>, in that the events occurred mainly in the centromeric region (*b-cn*) and in the right arm (*cn-bw*). In addition, the distribution of recombination events does not fit a Poisson distribution (index of dispersion  $\chi^2_{51} = 163.5$ ,  $P < 0.001$ ). Some of these induced recombination events therefore occurred as clusters, suggesting a premeiotic origin. A similar observation has been made with *OK1/dp b cn bw* flies (unpublished results of R. C. W. and J. N. Thompson). It should also be noted that the factor(s) causing male recombination could be transmitted over at least one generation.

In a second experiment 1–2-d-old *CS/dp b cn bw*; *CS/ve* males were injected with an *OK1* ovarian extract prepared by dissecting 100 fertilised *OK1* ovaries into 0.1 ml of a 0.4% saline solution, homogenising and centrifuging at 14,500 r.p.m. for 12 min. Control males were injected with a similarly prepared *CS* ovarian extract, or were left uninjected. After 1 or 2 d, injected

Table 1 Frequencies of male recombination induced by *OKI* extract

| Experiment 1 (whole-fly homogenate)                      |                                         |            |            |                        |          | Injection of CS homogenate      |          |          |          |               | Injection of Ringer solution |          |          |          |               |          |
|----------------------------------------------------------|-----------------------------------------|------------|------------|------------------------|----------|---------------------------------|----------|----------|----------|---------------|------------------------------|----------|----------|----------|---------------|----------|
| Treatment                                                | Injection of <i>OKI</i> homogenate      |            |            |                        |          | A                               | B        | C        | D        | E             | A                            | B        | C        | D        | E             | F        |
| Injected ♀♀                                              | A                                       | B          | C          | D                      | E        | A                               | B        | C        | D        | E             | A                            | B        | C        | D        | E             | F        |
| No. of ♂♂ tested                                         | 5                                       | 9          | 5          | 16                     | 17       | 15                              | 15       | 15       | 15       | 2             | 15                           | 12       | 15       | 10       | 9             | 9        |
| No. of ♂♂ with recombination progeny                     | 0                                       | 1          | 1          | 1                      | 3        | 0                               | 0        | 0        | 0        | 0             | 0                            | 0        | 0        | 0        | 0             | 0        |
| Mean ± s.d. of progeny size                              | 328 ± 87                                | 347 ± 77   | 355 ± 175  | 275 ± 66               | 249 ± 56 | 191 ± 63                        | 209 ± 56 | 208 ± 56 | 196 ± 61 | 240 ± 29      | 193 ± 54                     | 221 ± 54 | 212 ± 67 | 229 ± 40 | 197 ± 31      | 214 ± 44 |
| No. of recombinations                                    | 0                                       | 4          | 2          | 5                      | 6        | 0                               | 0        | 0        | 0        | 0             | 0                            | 0        | 0        | 0        | 0             | 0        |
| Total no. of progeny                                     | 1,639                                   | 3,127      | 1,777      | 4,401                  | 4,237    | 2,865                           | 3,137    | 3,124    | 2,944    | 479           | 2,888                        | 2,653    | 3,178    | 2,291    | 1,774         | 1,929    |
| Frequency of recombination                               | 0%                                      | 0.13%      | 0.11%      | 0.11%                  | 0.14%    | 0                               | 0        | 0        | 0        | 0             | 0                            | 0        | 0        | 0        | 0             | 0        |
| Region of second chromosome where recombination occurred |                                         |            |            |                        |          |                                 |          |          |          |               |                              |          |          |          |               |          |
| <i>dp-b</i>                                              | -                                       | -          | -          | -                      | ♂1       |                                 |          |          |          |               |                              |          |          |          |               |          |
| <i>b-cn</i>                                              | -                                       | 4+ + cn bw | 2+ + cn bw | 1 dp b++               | -        | 2+ b cn bw                      |          |          |          |               |                              |          |          |          |               |          |
| <i>cn-bw</i>                                             | -                                       | -          | -          | { 2 dp b cn+<br>2+++bw | 1+++bw   | -                               |          |          |          |               | { 2 dp b cn+<br>1+++bw       |          |          |          |               |          |
| Overall frequency of recombination*                      |                                         |            |            | 17/15,181 = 0.11%†     |          |                                 |          |          |          | 0/12,549 = 0% |                              |          |          |          | 0/14,713 = 0% |          |
| Experiment 2 (ovarian extract)                           |                                         |            |            |                        |          | Injection of CS ovarian extract |          |          |          |               | Uninjected                   |          |          |          |               |          |
| Treatment                                                | Injection of <i>OKI</i> ovarian extract |            |            |                        |          | Injection of CS ovarian extract |          |          |          |               | Uninjected                   |          |          |          |               |          |
| No. of ♂♂ tested                                         | 50                                      |            |            |                        |          | 70                              |          |          |          |               |                              |          |          |          |               |          |
| No. of ♂♂ with recombination progeny                     | 4                                       |            |            |                        |          | 0                               |          |          |          |               |                              |          |          |          |               |          |
| Mean ± s.d. of progeny size                              | 160 ± 103                               |            |            |                        |          | 198 ± 74                        |          |          |          |               |                              |          |          |          |               |          |
| Region of second chromosome where recombination occurred |                                         |            |            |                        |          |                                 |          |          |          |               |                              |          |          |          |               |          |
| <i>dp-b</i>                                              | ♂1                                      |            |            |                        |          | ♂2                              |          |          |          |               | ♂3                           |          |          |          |               |          |
| <i>b-cn</i>                                              | 1+ + cn bw                              |            |            |                        |          | 3+ + cn bw                      |          |          |          |               | 2 dp b++                     |          |          |          |               |          |
| <i>cn-bw</i>                                             | -                                       |            |            |                        |          | -                               |          |          |          |               | -                            |          |          |          |               |          |
| Overall frequency of recombination*                      | 8/7,989 = 0.10%†                        |            |            |                        |          | 0/13,856 = 0%                   |          |          |          |               | 0/26,792 = 0%                |          |          |          |               |          |

\*The frequencies of male recombination among males that showed recombination in experiment 1 was 17/1845 = 0.92%, and in experiment 2, 8/1135 = 0.71%.

†These results are significantly different from the controls at the 0.1% level by Fisher's exact test.



males were mated individually to *dp b cn bw*; *ve* virgin females for 4 d and then were remated every 3 d to fresh *dp b cn bw*; *ve* virgin females for five broods. The progeny were scored for second chromosome male recombination.

The results (summarised in Table 1), support those of the first experiment, and suggest that the factor(s) can be transferred by ovarian extracts.

As a further statistical test on the combined data from this study, a Fisher's exact test was performed on the number of OKI extract-treated males that produced any recombinant progeny (10/102) compared with the corresponding number of control males (CS and Ringer-injected) (0/202). These results show that significantly more treated males showed recombination than did control males ( $P = 0.000025$ ).

The results of the previous attempts<sup>4-6</sup> to induce male recombination by ovarian extract injections can be explained if Reddi *et al.*<sup>4</sup> injected an ovarian extract from flies showing male recombination, and the others<sup>5,6</sup> did not. Further experiments are planned to investigate the nature of the male recombination-inducing factor in the extracts of OKI flies.

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*Note added in proof:* Slatko and Hiraizumi (personal communication), using a *D. melanogaster* line that contains the *Male recombination* element, have observed results similar to those in this article.

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## Initiation of meiotic maturation in *Xenopus laevis* oocytes by lanthanum

PROGESTERONE can induce true maturation of isolated amphibian oocytes *in vitro*<sup>1</sup>. It has been found recently that the ionophore A23187, in the presence of high magnesium or calcium concentrations, can also promote meiosis *in vitro*<sup>2</sup>, and it has been observed that 0.5-1 mM propranolol and some related compounds, are potent inducers of maturational events in *Xenopus laevis* oocytes<sup>3</sup>. This last result suggests that displacement of membrane calcium might be involved in maturation, because this is known to occur in other systems treated with high concentrations of propranolol<sup>4</sup>. We have therefore studied the action of  $\text{La}^{3+}$ , since this ion is expected to act specifically at the superficial plasma membrane calcium sites<sup>5</sup>. We report here that maturation is induced by  $\text{La}^{3+}$ , indicating strongly that calcium ion movements have a role in the initiation of oocyte maturation.

Ovarian tissues were removed from *Xenopus laevis* females through a small incision made on the lateral abdominal wall. To obtain fully grown oocytes free of follicle cells, the ovaries were treated with collagenase<sup>6</sup>. In each experiment, all oocytes were obtained from the same animal. The experiments were conducted at room temperature, in a modified Ringer solution to which  $\text{La}^{3+}$  was added as the

**Table 1** Maturation of *Xenopus laevis* oocytes, as indicated by germinal vesicle breakdown, after continuous exposure to lanthanum or progesterone

| Treatment                                                             | A  | Female B | C  |
|-----------------------------------------------------------------------|----|----------|----|
| Modified Ringer                                                       | 0  | 0        | 0  |
| $\text{La}^{3+}$ (mM)                                                 |    |          |    |
| 0.01                                                                  | —  | —        | 0  |
| 0.1                                                                   | —  | —        | 2  |
| 0.5                                                                   | —  | —        | 3  |
| 1                                                                     | 0  | —        | 5  |
| 2.5                                                                   | 50 | —        |    |
| 5                                                                     | 60 | 55       | 72 |
| 10                                                                    | 75 | 62       | 92 |
| $\text{La}^{3+}$ (mM)<br>+ cycloheximide (100 $\mu\text{g ml}^{-1}$ ) | 10 | 0        | 0  |
| Progesterone (1 $\mu\text{g ml}^{-1}$ )                               | 97 | 100      | 91 |

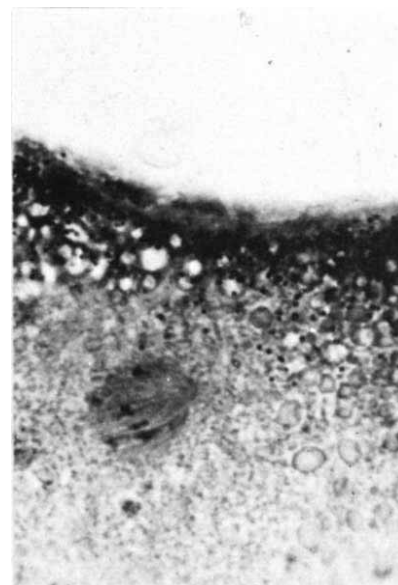
One hundred isolated oocytes were used for each set of conditions. The table shows the number of matured oocytes after 15 h incubation.  $\text{La}^{3+}$  was administered in solution as the chloride salt in Ringer solution containing no bicarbonate buffer.

—, Not tested.

chloride salt.  $\text{La}^{3+}$  at 5 or 10 mM promotes maturational events in *Xenopus laevis* oocytes (Table 1). The maturation is not abortive, since microscopic examination reveals the existence of the spindle moving to the cortex (Fig. 1).  $\text{La}^{3+}$ -induced maturation presents several other characteristics common to progesterone-induced maturation. The breakdown of the germinal vesicle takes place after about the same length of incubation, and maturational responses to both agents are inhibited by cycloheximide (Table 1; ref. 7);  $\text{La}^{3+}$ , like progesterone, is not able to induce maturation when injected into the oocytes (data not shown).

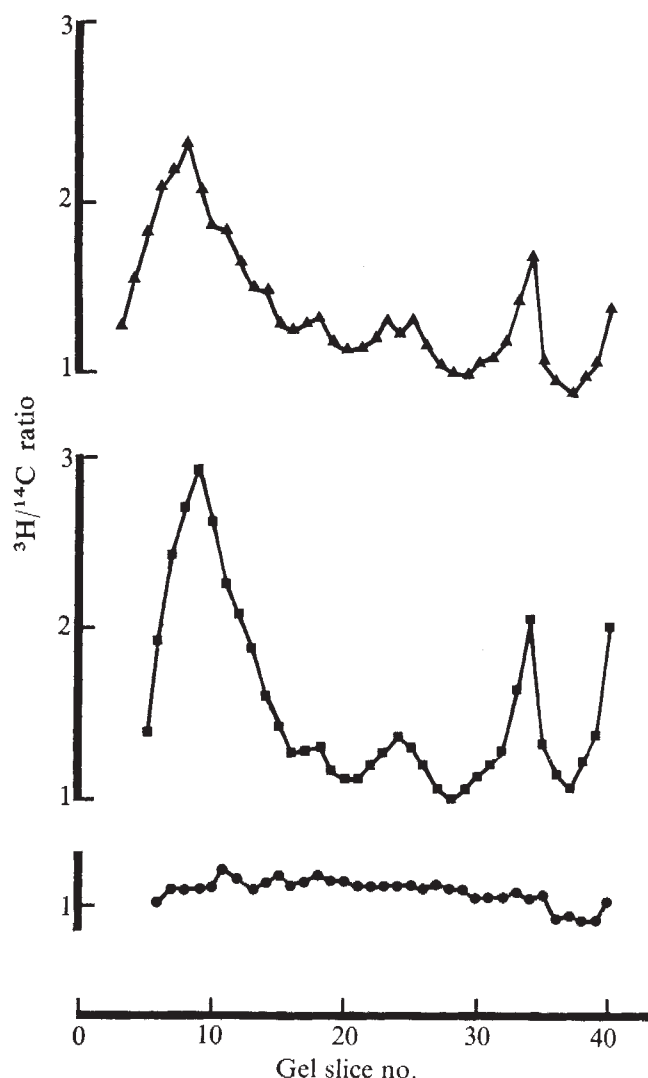
Furthermore, a similar preferential protein synthesis is activated by progesterone and  $\text{La}^{3+}$ . It has been shown previously that during progesterone-induced maturation *in vitro*, several electrophoretically defined soluble proteins are increased selectively<sup>8</sup>. We studied this effect by double labelling of proteins, injecting  $^3\text{H}$ -leucine into treated oocytes (maturing) and  $^{14}\text{C}$ -leucine into control oocytes (resting)

**Fig. 1** Meiotic metaphase of oocyte in which maturation was induced *in vitro* by  $\text{La}^{3+}$ , at 5 mM (chloride salt). Magnification  $\times 800$ . The oocyte was fixed in Smith's solution. Sections (7  $\mu\text{m}$ ) were stained with Feulgen's reagent and counterstained with light-green.



(Fig. 2). Control/control experiments with injection of  $^3\text{H}$ - and  $^{14}\text{C}$ -leucine into non-treated oocytes did not show any peaks, and the  $^3\text{H}/^{14}\text{C}$  ratio of electrophoresed proteins was normalised to 1. The same pattern of induced proteins was observed when  $\text{La}^{3+}$  was substituted for progesterone (Fig. 2). In each case, two distinct peaks were found, with several smaller peaks in between. For both these agents, the increase above the control  $^3\text{H}/^{14}\text{C}$  ratio was 150–200% for the small

**Fig. 2** Normalised gel electrophoresis patterns of proteins during maturation induced by progesterone ( $\blacktriangle$ ) and  $\text{La}^{3+}$  ( $\blacksquare$ ). The curves show  $^3\text{H}/^{14}\text{C}$  ratios, expressed as the percentage increase above the controls ( $\bullet$ ). Labelling was for 2 h beginning approximately 15 h after continuous exposure to progesterone or  $\text{La}^{3+}$ . Twenty-five matured oocytes were injected with 70 nl of  $^3\text{H}$ -Leu and 25 non-treated oocytes were injected with 70 nl of  $^{14}\text{C}$ -Leu. Control/control ( $\bullet$ ) was performed by injecting 25 non-treated oocytes with  $^3\text{H}$ -Leu and 25 other non-treated oocytes with  $^{14}\text{C}$ -Leu ( $^3\text{H}$ -Leu, 55 Ci mmol $^{-1}$ ;  $^{14}\text{C}$ -Leu, 324 mCi mmol $^{-1}$ , Amersham). Appropriate amounts of non-radioactive amino acids were added to obtain identical concentrations of  $^3\text{H}$ - and  $^{14}\text{C}$ -amino acids and a  $^3\text{H}/^{14}\text{C}$  d.p.m. ratio of about 10. Treated and control oocytes were pooled and homogenised in 2 ml of saline solution. The 105,000g supernatant was obtained and dialysed as reported previously<sup>8</sup>. Sodium dodecyl sulphate (0.1%)–polyacrylamide gel (6%) electrophoresis was performed under 2 mA per tube for 20 min and then 5 mA per tube (migration towards anode on the left). Gels were re-electrophoresed in 7% acetic during 2 h and sliced immediately. Soluene (0.5 ml) was added and the supernatant left for 3 h at 50 °C. Radioactivity was then counted in an appropriate scintillation mixture. Left peak, histones migrate at this level; right peak (higher molecular weight), molecular weight greater than 100,000. Other details are in ref. 12.



molecular weight peak and 130–150% for the large molecular weight peak.

Incubation in  $\text{Ca}^{2+}$  alone in high concentration does not lead to maturation<sup>2</sup>. Although ionophore A23187 induces maturation in amphibian oocytes given certain conditions of divalent ionic concentration<sup>2</sup>,  $\text{La}^{3+}$  seems to be a more appropriate agent to investigate the possible involvement of  $\text{Ca}^{2+}$  in this, and other systems<sup>3,9</sup>. High concentrations of extracellular  $\text{La}^{3+}$  displace external membrane calcium, and block both  $\text{Ca}^{2+}$  uptake and efflux, resulting in a release of internal sequestered  $\text{Ca}^{2+}$  (ref. 5). Our finding that  $\text{La}^{3+}$  induces maturation makes it likely that  $\text{Ca}^{2+}$  is involved in the initiation of maturation. Since injected  $\text{Ca}^{2+}$  is without effect<sup>10</sup>, however, it must be that the  $\text{Ca}^{2+}$  movements involved are highly regulated, or need to be sustained during the period of maturation. Injected  $\text{La}^{3+}$  is without effect, probably because it is unable to react with the appropriate membrane sites.

The present results imply that progesterone, the likely physiological inducing agent<sup>11</sup>, acts at specific membrane sites, leading to well defined movements of  $\text{Ca}^{2+}$  inside the cell. The altered  $\text{Ca}^{2+}$  distribution would in turn be the intermediary step for the series of further events promoting maturation.

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## Benzpyrene induces sister chromatid exchanges in cultured human lymphocytes

A WIDE variety of chemical mutagen/carcinogens induce sister chromatid exchanges (SCE) in cultured Chinese hamster cells and can thus be studied at the chromosomal

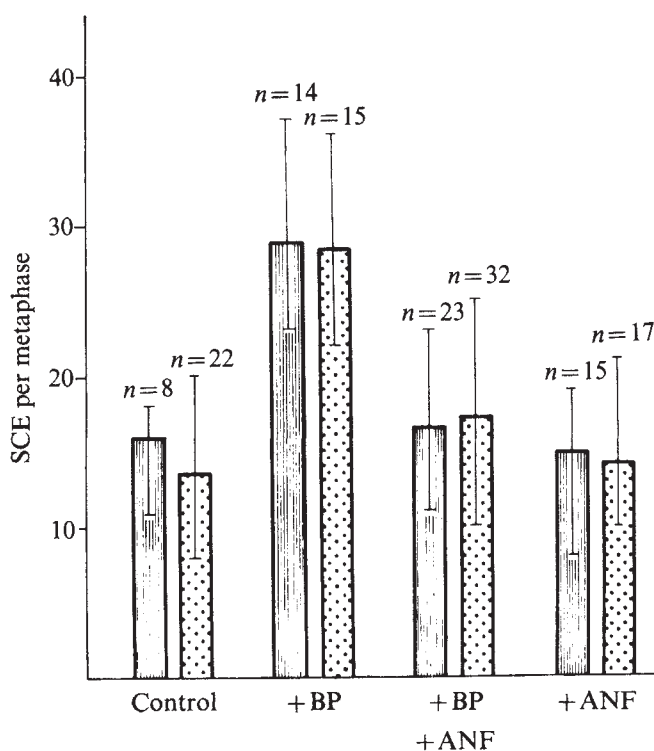
level<sup>1,2</sup>. Certain polycyclic hydrocarbons, such as benzo(a)-pyrene, 3-methylcholanthrene, dimethylbenzanthracene and others are important in the aetiology of human bronchial carcinoma<sup>3</sup>. The substances are not themselves carcinogenic, but are converted to carcinogenic epoxides by the enzyme arylhydrocarbonhydroxylase.<sup>4</sup> Epoxides bind non-enzymatically to DNA, unless they are converted enzymatically to inert water-soluble metabolites by epoxide hydratase<sup>5</sup> and glutathione-S-transferase<sup>6</sup>. The carcinogenic effect of polycyclic hydrocarbons is closely connected with the regulation of the enzyme activities and genetic variability can thus be expected. It has been observed at the population level by family studies<sup>7</sup> and at the cellular level by biochemical differences in the hydrocarbon-degrading enzyme system<sup>8</sup>. We have investigated the possible induction of sister chromatid exchanges by benzo(a)pyrene and show that the rate of SCE does not correlate with different rates of benzpyrene metabolism. We therefore suggest that genetic differences in benzpyrene metabolism are unrelated to genetic variation in the predisposition to bronchial carcinomas.

We tested the intracellular benzo(a)pyrene (BP) metabolism by measuring the formation of tritiated H<sub>2</sub>O-soluble products from <sup>3</sup>H-BP in cultured lymphocytes isolated by a Ficoll-Hypaque gradient as described in Fig. 1. An inhibitor of the monooxygenase system<sup>9</sup>, alpha-naphthylflavone (ANF, 55 µg ml<sup>-1</sup>) was used as a control. We found wide individual differences in BP metabolism among 22 individuals. The one with the highest metabolic activity (WW) showed 3.8 ± 0.5 times the activity of the lowest (HR) in each of six independent determinations. ANF reduced the amount of water-soluble products to background levels in both.

To determine sister chromatid exchange rate (SCE), 10<sup>6</sup> isolated lymphocytes were incubated in 5 ml of chromosome medium 1a (Gibco) together with 5-bromodeoxyuridine (BUDR) (30 µg ml<sup>-1</sup>) for 70 h at 37 °C in the dark. Parallel assays were made in duplicate with 10<sup>-6</sup> M BP and 55 µg ml<sup>-1</sup> ANF. Chromosomes were prepared by standard techniques and the slides were stained with acridine orange (125 µg ml<sup>-1</sup>) in phosphate buffer at pH 6.0 and analysed by fluorescence microscopy.

The results are summarised in Fig. 1. The SCE rate nearly doubled after incubation with BP, but not in the presence of ANF (55 µg ml<sup>-1</sup>). This concentration inhibits the formation of water-soluble products by more than 95%. Benzpyrene failed to induce SCEs when it was present for only 30 h of culture, instead of the usual 70 h, and it did not influence the total number of second generation metaphases found at 70 h. The fast (WW) and the slow (HR) BP-metabolising individuals did not differ in the rate of SCE induction.

This result, at first surprising, corresponds to differences in BP metabolism between different strains of mice which, nevertheless, do not differ in the rate of benzpyrene-induced tumours<sup>10,11</sup>. The inhibition of the first BP-oxidising step (AHH) by ANF prevents the carcinogenic effect of BP as indicated by the normal SCE induction. The similar SCE induction between the high (WW) and the low (HR) BP-metabolising individuals (Fig. 2) indicates, however, that BP metabolism and carcinogenesis are not related in a simple, proportional way<sup>12</sup>. A high rate of benzpyrene conversion cannot serve as an indication of the effective concentration of carcinogenic epoxides and their effects on DNA. The formation of water-soluble products from BP only indicates that BP is being degraded, but does not give any information about the current levels of ultimate carcinogens formed during this steady-state process. How much epoxide binds to DNA is also unknown. Finally, we do not know how much epoxide is removed by DNA repair and how much remains fixed in the DNA following the next replication.



**Fig. 1** Induction of sister chromatid exchanges (SCE) by benzpyrene (BP) and its inhibition by alpha-naphthylflavone in lymphocyte cultures with different rates of intracellular BP metabolism. Lymphocytes were isolated from 20 ml of heparinised blood by a Ficoll-Hypaque gradient<sup>13</sup> which yielded a 70–85% pure preparation of about  $2 \times 10^7$  lymphocytes. The BP metabolism was determined by seeding  $5 \times 10^5$  cells into each of 96 wells of a Cook's microtitre plate (Falcon, Oxnard, California) together with 200 µl of Ham's F-12 culture medium containing 16% foetal calf serum and 150 nM of <sup>3</sup>H-BP (specific activity 25 Ci mmol<sup>-1</sup>, Amersham-Buchler, Braunschweig, Germany). Water-soluble products were determined after 8 h of incubation by the method of Huberman and Sachs<sup>14</sup>. SCEs were scored in phytohaemagglutinin-stimulated lymphocytes after 70 h of incubation at 37 °C. The height of the columns (shaded, fast; dotted, slow, BP-metabolising individual) indicate the mean and the bars the range of SCE per metaphase. Benzo(a)pyrene (Sigma) was dissolved in chloroform : methanol (2:1), extracted twice by equal amounts of 1 N NaOH and recrystallised from the organic phase in the cold. A stock solution (10 mM) of BP in acetone was mixed with a 40-fold amount of foetal bovine serum and added to the series indicated to give a final BP concentration of 1 µM. The monooxygenase inhibitor ANF (Fluka) was also first dissolved in acetone mixed with foetal bovine serum and added where indicated at a final concentration of 55 µg ml<sup>-1</sup>. The rate of SCE induction in the presence of the inhibitor does not differ from cultures without BP or controls. There is only a minimal SCE induction by BP, in the presence of ANF, probably as a result of incomplete inhibition of the monooxygenase system. At the concentration used, formation of water-soluble products was less than 5% of controls without ANF.

The induction of SCEs by benzpyrene reported here probably reflects the intracellular carcinogenic effect of benzpyrene, although it is not understood in molecular terms. The lack of correlation between the activity of intracellular benzpyrene metabolism and SCE rate suggests that genetic differences in BP metabolism are not related to genetic differences in the predisposition towards development of bronchial carcinoma as revealed by family studies<sup>7</sup>. How the rate of benzpyrene-induced sister chromatid exchanges differs between patients with bronchial carcinoma and control patients is being investigated.



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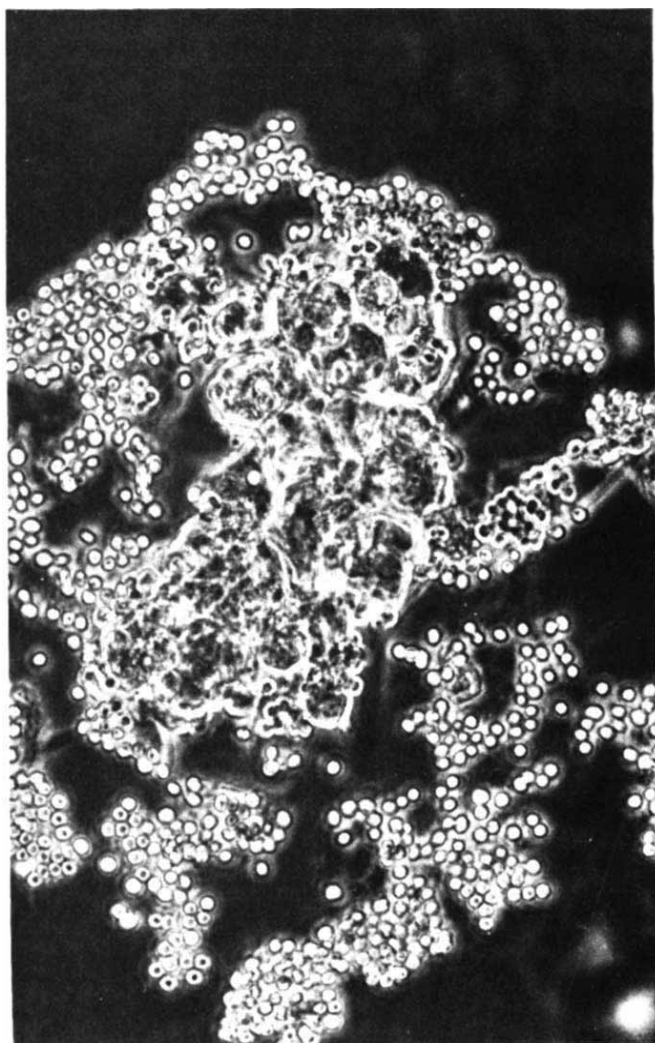
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## Expression of maternal and paternal antigens on trophoblast

GENETICALLY different individuals will not usually accept tissue grafts from one another. The best known exception to this is the ability of the foetus to act as a homograft. Fertilised mouse eggs contain both paternally and maternally derived antigens<sup>1,2</sup>, but it has not so far been demonstrated whether paternal antigens are expressed on mouse trophoblast, the embryo tissue which forms initial contact with the maternal circulation. If present, such antigens may be masked by a mucopolysaccharide<sup>3</sup>; this has been disputed by Simmons *et al.*<sup>4</sup>. Alternatively, it has been proposed that trophoblast lacks transplantation antigens<sup>5</sup> and Searle *et al.*<sup>6</sup> failed to immunise a recipient with mouse trophoblast when tested either by skin grafting or cell-mediated cytotoxicity. It has been suggested, however, that histocompatibility antigens on human trophoblast were responsible for cytotoxic antibody found in the sera of 12-week human pregnancies<sup>7</sup>, and Nakakita showed antibody production in mice to human trophoblast, using immune adherence<sup>8</sup>. I now report the presence of both paternal and maternal antigens on trophoblast outgrowths of mouse blastocysts *in vitro*.

Blastocysts flushed from the uteri of either CBA/Fa or C57 BL/Mcl females 3.5 d after coitus were cultured on glass coverslips. On day 4 of culture, the mixed immune haemadsorption technique<sup>10</sup> (MHA) modified by Tachibana *et al.*<sup>11</sup> was used to detect antigens on the outgrowths. Trophoblast and indicator cells (sheep red blood cells) were coated with specific antisera raised in mice, and linked together by a rabbit anti-mouse IgG serum (Capell Laboratories). Photographs were taken as a cross check. Figure 1 shows a 3.0 reaction. The attachment of sensitised red cells to trophoblast outgrowths from crosses of (CBA × CBA), (C57BL × CBA) and (CBA × C57BL) incubated with anti-CBA serum (Fig. 2) shows that both maternal and

paternal antigens are expressed on the trophoblast. For each pair of crosses the difference between the mean degree of red-cell attachment was significant ( $P < 0.01$ ). This suggests that the amount of CBA antigen present is greater with a CBA mother than with a CBA father, and greater still when both parents are CBA. Control data for (CBA × CBA) trophoblast outgrowths are shown in Table 1. Normal CBA or C57BL sera and anti-C57BL serum gave scores of 0. After absorption



**Fig. 1** A (C57BL × CBA) blastocyst cultured in Snow's medium<sup>9</sup> and 10% foetal calf serum under oil and incubated at 37 °C in an atmosphere of 10% CO<sub>2</sub> in air. Attachment of sensitised red cells to the resulting outgrowth after MHA is shown. Antiserum to a mixture of male and female CBA/Fa spleen cells was raised in C57BL/Mcl mice. Approximately half a donor spleen was given to each recipient weekly for between 7 and 10 weeks. Antiserum to the sheep erythrocytes was raised in A-strain mice. Recipients were given 0.2 ml of a 1% suspension of sheep red blood cells (SRBC) each week for between 7 and 10 weeks. Optimal conditions for the sensitisation of SRBCs were established. After washing three times in phosphate-buffered saline (PBS) they were incubated in a 1:1,000 dilution of anti-SRBC serum followed by a 1:100 dilution of anti-mouse IgG serum and finally diluted to 4% (v/v) and stored at 4 °C. There were three washes with PBS between each step in the procedure. On day 4, outgrowths on coverslips were rinsed in PBS and incubated for 1 h with selected antisera, rinsed and layered with sensitised SRBCs (1.0% v/v). After 1 h at room temperature, unattached erythrocytes were washed off and the strength of the reaction was estimated microscopically according to the percentage of target cells (that is, trophoblast of the outgrowth) to which sensitised SRBCs were attached, expressed on the following scale: 4.0 (100% attachment); 3.5 (90%); 3.0 (50–80%); 2.0 (<50%); 1.0 (<25%); 0.5 (<10%); 0 (0%). The above outgrowth was given a score of 3.0.

**Table 1** Expression of CBA cell-surface antigens on trophoblast outgrowths of blastocysts from a CBA × CBA mating after incubation with control sera

| Serum        | Cell type used for absorption | Serum dilution | Mean score ± s.e.m. |      |
|--------------|-------------------------------|----------------|---------------------|------|
| Anti-CBA     | —                             | 1:160          | 3.80 ± 0.06         | (34) |
| Anti-CBA     | CBA liver and spleen          | 1:160          | 2.00*               | (3)  |
| Anti-CBA     | C57BL liver and spleen        | 1:160          | 3.75 ± 0.25         | (4)  |
| Anti-CBA     | CBA lymph node and spleen     | 1:160          | 0.52 ± 0.07*        | (24) |
| Anti-CBA     | C57BL lymph node and spleen   | 1:160          | 3.84 ± 0.05         | (29) |
| Normal CBA   | —                             | 1:40           | 0                   | (13) |
| Normal C57BL | —                             | 1:40           | 0                   | (10) |
| Anti-C57BL   | —                             | 1:40           | 0                   | (20) |

Absorptions were carried out with a 50:50 mixture of washed liver and spleen cells or lymph node and spleen cells for 2 × 1 h at 37 °C. Results are expressed as mean score ± s.e.m. The number of outgrowths per group is in parentheses.

\**P* < 0.001 for corresponding controls.

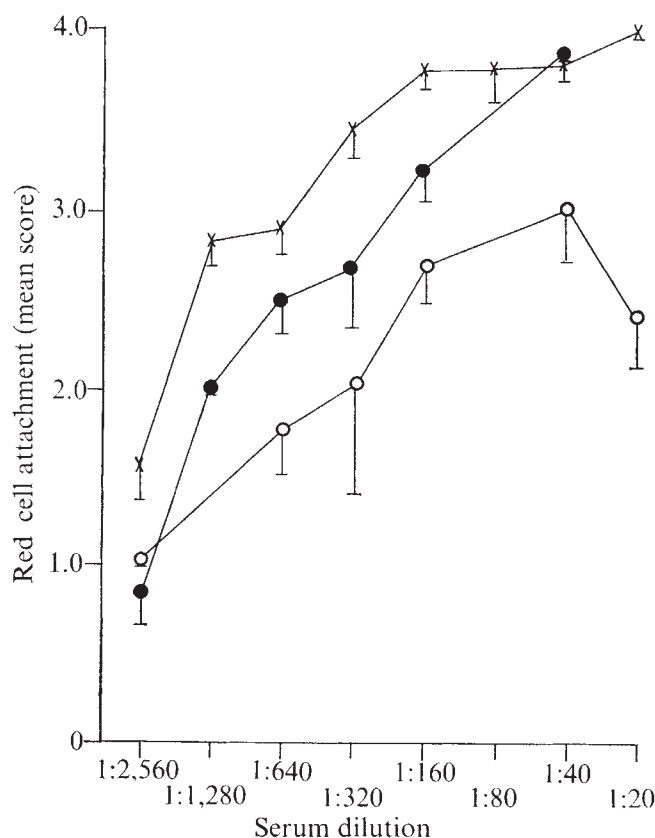
**Table 2** Expression of C57BL cell-surface antigens on trophoblast outgrowths after incubation with normal sera, anti-C57BL serum, or absorbed anti-C57BL serum, diluted 1:40

| Origin of blastocysts<br>Mother Father | Anti-C57BL serum | Anti-C57BL serum absorbed with C57BL cells | Anti-C57BL serum absorbed with CBA cells | Normal C57BL serum | Normal CBA serum | Anti-CBA serum 1:160 |
|----------------------------------------|------------------|--------------------------------------------|------------------------------------------|--------------------|------------------|----------------------|
| C57BL × C57BL                          | 3.86 ± 0.14 (4)  | 1.00 ± 0.14* (10)                          | 3.38 ± 0.34 (8)                          | 0 (4)              | 0 (4)            | 0 (10)               |
| CBA × C57BL                            | 3.31 ± 0.23 (13) | 1.00 (2)                                   | 3.00 (2)                                 | —                  | —                | —                    |
| C57BL × CBA                            | 3.00 (2)         | 0.89 ± 0.07* (9)                           | 3.00 ± 0.37 (6)                          | —                  | —                | —                    |

Absorptions were carried out with a 50:50 mixture of washed spleen cells and lymph node cells for 2 × 1 h at 37 °C. Results are expressed as mean score ± s.e.m. The number of outgrowths per group is in parentheses.

\**P* < 0.001 for corresponding controls.

**Fig. 2** The expression of CBA cell surface antigens on trophoblast outgrowths after incubation with serial dilutions of anti-CBA serum. Blastocysts were recovered from CBA females mated to CBA (×) or C57BL (●) males, and from C57BL females mated to CBA males (○). Data were accumulated from 14 experiments. A least squares analysis of variance according to the method of fitting constants<sup>12</sup> gave a mean square value of 0.2623 with 152 residual degrees of freedom. Duncan's new multiple range test<sup>13</sup> was used to analyse the difference between the means of the three crosses (*P* < 0.01).



of the anti-CBA serum with a mixture of spleen and lymph node cells from CBA mice, red-cell attachment was reduced by 86%. Total absorption of the activity was not expected because the antiserum was made to both intracellular and cell membrane-bound antigens of spleen cells, whereas the absorption would only remove antibodies raised against the latter. A mixture of CBA liver and spleen cells only reduced the reaction by 50%. Absorption with C57BL cells had no effect.

Table 2 shows results obtained with blastocysts from (C57BL × C57BL), (CBA × C57BL) and (C57BL × CBA) matings incubated with anti-C57BL serum and control sera. Both maternal and paternal antigens are present but a comparison of their relative expression cannot be made because of the small number of outgrowths studied when the C57BL is the mother.

These experiments show that trophoblast *in vitro* exhibits antigenicity derived from both the maternal and paternal genome. The antigens expressed could be either major histocompatibility antigens of the H-2 type or minor antigens (for example, H-3, H-6), since both are present on spleen cells.

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## Successful induction of specific tolerance to transplantation antigens using autoimmunisation against the recipient's own natural antibodies

ORGAN transplantation, especially of kidneys, is now an accepted method in medical care. In spite of careful matching to obtain the most suitable combination between donor and recipients as to blood group and HLA antigens, varying degrees of incompatibility have to be accepted. This makes prolonged use of immunosuppressive drugs necessary. Such therapy, however, may in itself cause serious and even lethal complications. Specific tolerance to the donor antigens with full reactivity against other antigens is the ideal. Classical induction of transplantation tolerance requires massive inoculation of foreign cells, may result in serious graft-versus-host reactions and is not considered feasible in human kidney grafts. We describe here a new method of inducing specific immune tolerance to a given set of alloantigens, which can be carried out in adults and which will leave reactivity against other antigens intact.

Every individual seems to have lymphocytes with predetermined receptors for various parts of the major histocompatibility complex antigens of the species. When tested against a given set of such MRC antigens, 3–10% of a normal mouse or rat T-lymphocyte population will express immune competence<sup>1,2</sup> or specific receptors for antigens of that particular MHC locus<sup>3,4</sup>. These receptors can be characterised according to two characteristics, their specific antigen-binding capacity and their unique antigenic or idiotypic markers<sup>5,6</sup>. Two other facts make these receptors useful for the induction of specific immune tolerance. They are found in detectable amounts in normal serum or urine of the individual<sup>7</sup>. It can be shown that the individual is able to produce specific anti-receptor antibodies against its own naturally occurring receptors, if the latter are presented to the individual in a concentrated, modified form. We describe results showing the validity of the latter statement.

Normal Lewis rats (MHC type Ag-B<sup>1</sup>) have in their sera or urine idiotype receptor molecules with specificity for other Ag-B alloantigens, e.g. against DA (Ag-B<sup>4</sup>) and BN (Ag-B<sup>3</sup>). Using anti-(Lewis-anti-DA) idiotype antibodies linked to Sepharose it is possible to isolate the anti-DA receptors from normal Lewis serum<sup>7</sup>. Normal Lewis serum (300 ml) was thus passed over such an anti-idiotype column and the bound material was acid eluted and cross linked with glutaraldehyde<sup>8</sup>. Cross-linked material (20 µg) in Freund's complete adjuvant was injected subcutaneously into each of four normal adult Lewis rats. The animals received a boost on day 21. On day 40 the animals were bled and their blood lymphocytes used as responder cells in MLC tests against DA or BN. Four days

**Table 2** Delayed rejection of DA skin on immunised Lewis rats

| Immunised Lewis rats No. | Rejection of DA skin on day | Mean | Rejection of BN skin on day | Mean |
|--------------------------|-----------------------------|------|-----------------------------|------|
| 1                        | 21                          |      | 12                          |      |
| 2                        | 35                          |      | 11                          |      |
|                          |                             | 30.3 |                             | 11.8 |
| 3                        | 27                          |      | 11                          |      |
| 4                        | 38                          |      | 13                          |      |

Three control normal Lewis rats rejected DA skin between day 9 and 10 and BN skin between day 10 and 12. Full thickness skin grafts were carried out as described elsewhere<sup>10</sup>.

later the animals received DA skin grafts on the left back side and BN grafts on the right side.

As shown in Table 1, these Lewis rats showed a specific lack of reactivity against DA alloantigens in the MLC and they accepted DA skin grafts for some 30 d (See Table 2). Reactivity against BN alloantigens was in both tests completely normal. In three similar experiments with skin grafts essentially the same results were obtained. The mean survival of DA or (Lewis × DA)F<sub>1</sub> skin grafts on 20 adult Lewis rats treated in the above way was 24 ± 1.7 (s.e.) days. BN or (Lewis × BN)F<sub>1</sub> grafts were all rejected within 11 d.

On day 91 the animals were bled again and their sera tested, after extensive absorption with (L × DA)F<sub>1</sub> lymphocytes in order to remove possible anti-DA alloantibodies produced after skin rejection, for the ability to bind to normal Lewis or DA T lymphocytes using as second layer <sup>125</sup>I-protein A as an indicator for surface-bound IgG antibodies<sup>5</sup>. All sera contained antibodies capable of reacting with normal Lewis lymphocytes but not with DA lymphocytes. To prove the auto-anti-idiotype nature of these autoantibodies the sera were now tested for their ability to cause specific inhibition of MLC as had previously been done using "conventional" anti-idiotype antibodies<sup>5</sup>.

As shown in Table 3, treatment of normal Lewis T lymphocytes with the antisera in the presence of complement did result in a highly reduced activity against DA but not against BN alloantigens in the MLC test. Thus, the autoantisera did indeed contain the expected anti-idiotype antibodies.

Finally, on day 91 after the bleeding, each of the animals was inoculated with 2.5 × 10<sup>7</sup> (Lewis × DA)F<sub>1</sub> and (Lewis × BN)F<sub>1</sub> lymphocytes. Animals were killed 10 d later and their antisera were tested for alloantibodies using the protein A test. Then weak alloantibody titres against DA alloantigens could be detected in the autoimmunised animals, ranging from 1:32 to 1:256. Whether these alloantibodies contained the "normal" Lewis-anti-DA idiotypes was not tested. The control animals treated in exactly the same way as the experimental animals except for the immunisation with idiotype receptors had anti-DA titres ranging from 1:4,096 to 1:16,384. Both groups of animals had similar titres against BN alloantigens (range 1:2,048 to 1:4,096). Thus, not only did the auto-anti-idiotype

**Table 1** Lack of reactivity in MLC of autoimmune Lewis rats against DA alloantigens

| Responder cells from immunised Lewis rats No. | <sup>3</sup> H-TdR incorporation in mixture against DA. Mean c.p.m. of duplicates ± s.e. | <sup>3</sup> H-TdR incorporation in mixture against BN. Mean c.p.m. of duplicates ± s.e. | <sup>3</sup> H-TdR incorporation of responder cells alone. Mean c.p.m. of duplicates ± s.e. |
|-----------------------------------------------|------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|
| 1                                             | 9,141 ± 1,124                                                                            | 40,343 ± 7,927                                                                           | 7,875 ± 1,131                                                                               |
| 2                                             | 10,227 ± 610                                                                             | 45,663 ± 18,925                                                                          | 8,410 ± 424                                                                                 |
| 3                                             | 11,910 ± 2,429                                                                           | 38,841 ± 8,850                                                                           | 9,495 ± 619                                                                                 |
| 4                                             | 9,723 ± 3,714                                                                            | 42,346 ± 1,371                                                                           | 9,667 ± 224                                                                                 |
| Responder cells from normal Lewis rat         | 139,464 ± 15,855                                                                         | 39,271 ± 5,808                                                                           | 5,336 ± 455                                                                                 |

The MLC was performed in flat-bottom microtitre plates in EHAA medium<sup>9</sup> complemented with the double amount of glutamine and 0.5% fresh normal BN rat serum using 0.25 × 10<sup>6</sup> responder cells and 0.5 × 10<sup>6</sup> spleen cells irradiated with 2,000 R as stimulator cells. Cultures were collected after 120 h after a 6-h pulse with 1 µCi of <sup>3</sup>H-TdR (ref. 5). Stimulator cells alone gave between 200 and 400 c.p.m.



**Table 3** Serum from immunised Lewis animals specifically inhibits MLC

| Lewis responder cells treated with serum and complement: Serum from immunised Lewis rats | <sup>3</sup> H-TdR incorporation* of mixture against DA. Mean c.p.m. of triplicates $\pm$ s.e. | <sup>3</sup> H-TdR incorporation* of mixture against BN. Mean c.p.m. of triplicates $\pm$ s.e. | <sup>3</sup> H-TdR incorporation of responder cells alone. Mean c.p.m. of duplicates $\pm$ s.e. |
|------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| No.                                                                                      |                                                                                                |                                                                                                |                                                                                                 |
| 1                                                                                        | 32,344 $\pm$ 6,801 (43%)                                                                       | 31,179 $\pm$ 2,601 (89%)                                                                       | 3,684 $\pm$ 567                                                                                 |
| 2                                                                                        | 32,151 $\pm$ 3,139 (43%)                                                                       | 26,809 $\pm$ 2,428 (77%)                                                                       | 3,740 $\pm$ 563                                                                                 |
| 3                                                                                        | 20,837 $\pm$ 5,501 (28%)                                                                       | 34,537 $\pm$ 2,186 (99%)                                                                       | 3,996 $\pm$ 226                                                                                 |
| 4                                                                                        | 35,512 $\pm$ 3,851 (47%)                                                                       | 38,172 $\pm$ 2,703 (110%)                                                                      | 3,860 $\pm$ 333                                                                                 |
| Normal Lewis serum pool                                                                  | 75,456 $\pm$ 6,074 (100%)                                                                      | 34,855 $\pm$ 4,374 (100%)                                                                      | 3,457 $\pm$ 1,454                                                                               |

Conditions for the MLC essentially the same as in Table 1. For further details for the treatment of the responder cells with antiserum and complement see ref. 5. Sera from another group of immunised Lewis animals inhibited MLC completely in a specific way.

\*Values significantly different from values of normal Lewis serum and complement-treated groups are underlined.

reaction significantly and specifically wipe out the T-cell mediated reactivity against the relevant alloantigens but there was also a drastic reduction in the alloantibody producing ability of these individuals. Using lymphocytes from auto-idiotype-immunised rats we have also been able to demonstrate that they largely lack the corresponding idiotypes, for example Lewis rats immunised with Lewis-anti-DA receptors lack lymphocytes with idiotypes signifying anti-DA reactivity but show normal distribution of anti-BN idotype positive lymphocytes. Whether this reduction is particularly pronounced in the B- or the T-lymphocyte population is unknown.

We have thus presented data demonstrating that selective autoimmunity against a given group of antigen-binding receptors can lead to specific immune tolerance, probably due to the physical elimination of this group of immunocompetent cells. No side effects of this autoimmune procedure have so far been noted and the individuals express completely normal reactivity against other antigens. The skin-graft tolerance is highly significant; as in the rat many minor as well as the major Ag-B locus antigens do contribute to skin-graft rejection. In spite of this, grafts often survived for up to a month in the absence of any nonspecific immunosuppressive therapy. It thus seems clear that our techniques may allow the induction of long lasting immune tolerance through autoimmune reactions against the relevant idiotypic receptors. The specificity of this procedure would be as high as that of 'classical' immune tolerance and could in particular be carried out in adult, immunocompetent individuals. Improvements to make the approach more general by testing other species of animals and using alloantigenic immunosorbents instead of anti-idiotypic immunosorbents are required. Already at this early stage, however, it seems that autoimmune reactions can be used in a productive manner to manipulate the immune system in the transplantation immune system, in allergic disorders and perhaps even to regulate the course of certain autoimmune diseases.

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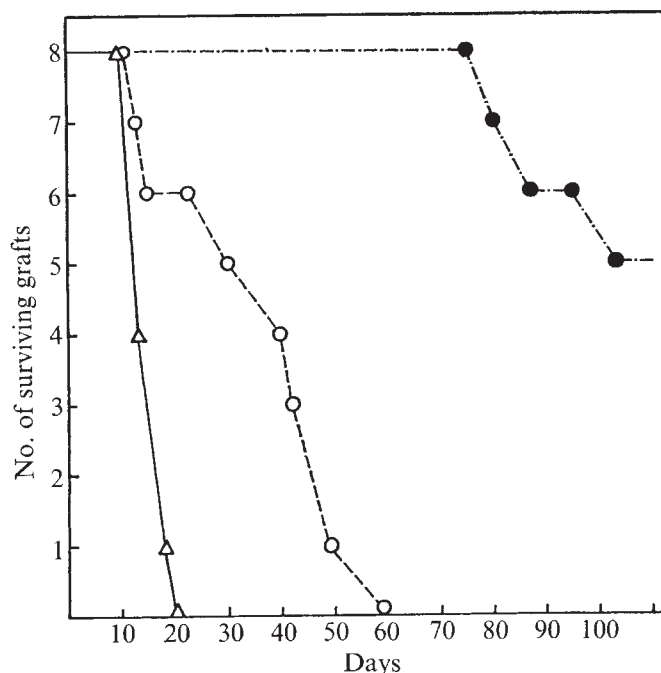
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## Induction of transplantation tolerance using serum as antigen source

It has been shown that massive doses of allogeneic serum produce the prolongation of renal allograft survival in adult pigs<sup>1</sup>. Furthermore, alloantigenic activity was demonstrated in human serum by inhibiting certain reactions *in vitro*<sup>2-6</sup>; and in some strains of mice, the presence of histocompatibility (H) antigens in the serum could be demonstrated by inhibition of the cytotoxicity of specific alloantisera<sup>7</sup>.

This study was designed to determine whether transplantation tolerance in rats can be induced by multiple massive doses of serum. Rats of the AVN strain were injected with allogeneic or syngeneic serum in increasing doses three times weekly from birth to the 6th week of age (1st week, 3  $\times$  0.3 ml; 2nd week, 3  $\times$  0.5 ml; 3rd week, 3  $\times$  1 ml; 4th and 5th weeks, 3  $\times$  1.5 ml; 6th week, 3  $\times$  2 ml). The donors of allogeneic sera were rats of the congenic strains Lewis (Lew) or Lew.1A, differing from the AVN strain recipients in, respectively, both H-1 plus non-H-1 antigens and in the non-H-1 antigens only. Four weeks after birth, animals were skin grafted. The group treated with Lew serum, the control groups treated with AVN serum and the group of untreated animals simultaneously received the

**Fig. 1** Survival of Lew.1A skin grafts after treatment with Lew.1A serum (●), AVN serum (○), untreated controls (Δ).



**Table 1** Testing of AVN anti-Lew cytotoxic antibodies in AVN rats injected with Lew or AVN serum from birth

| Group | Treatment | Serum titre* | Percentage $^{51}\text{Cr}$ release $\pm$ s.e.† |
|-------|-----------|--------------|-------------------------------------------------|
| 1     | —         | 1:8–1:64     | 28.8 $\pm$ 4.7                                  |
| 2     | AVN serum | 1:8–1:128    | 37.0 $\pm$ 12.6                                 |
| 3     | Lew serum | —            | 15.5 $\pm$ 1.9‡                                 |

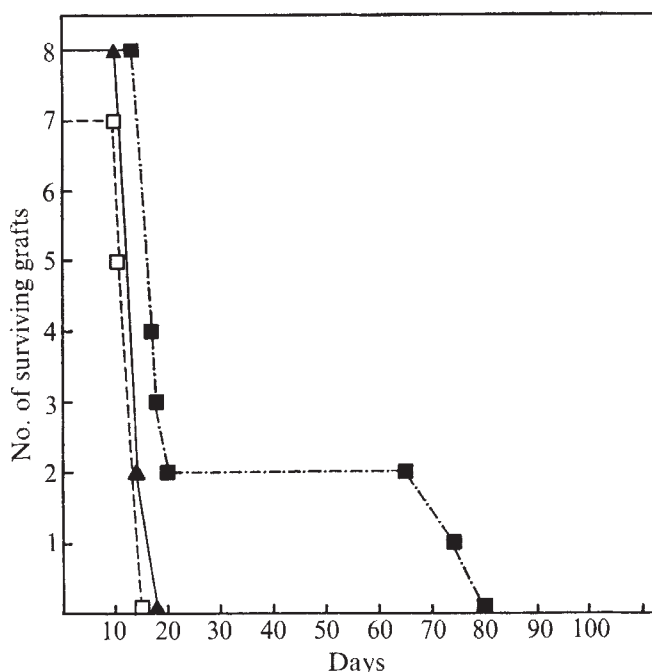
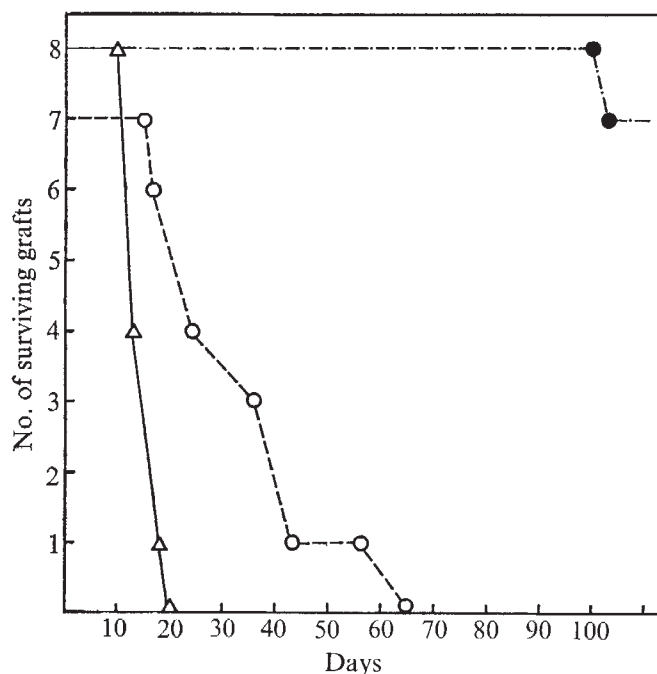
\*The cytotoxic activity of the serum was determined by the  $^{51}\text{Cr}$  release from labelled lymphocytes of the strain Lew by the standard technique<sup>8</sup>. The cytotoxic titre was determined as the highest serum dilution showing significantly higher  $^{51}\text{Cr}$  release than a similar dilution of normal serum.

†Percentage of released activity with a 1:1 dilution of serum.

‡Statistically significant ( $P < 0.001$ ) inhibition of cytotoxic antibody production in comparison with group 1 or group 2 and non-significant difference from the value of  $^{51}\text{Cr}$  release in the tests of serum from normal untransplanted rats ( $13.1 \pm 0.3\%$ ).

Lew and Lew.1A skin grafts. The group treated with Lew.1A serum and the corresponding control groups received only Lew.1A skin grafts.

A high degree of tolerance was obtained in the animals with the non-H-1 antigenic difference. Figure 1 shows the survival of skin grafts in animals treated with Lew.1A serum. All grafts survived 75 d and most of them more than 100 d. In addition to the specific antigenic activity of the serum, its nonspecific component(s) had some effect, as evident from the prolongation of skin graft survival in the control animals treated with syngeneic serum. Also, the Lew serum induced a high degree of tolerance of non-H-1 different Lew.1A grafts (Fig. 2). All these grafts survived 100 d and most of them persisted further. Again the nonspecific effect of the syngeneic serum was evident in the ability to prolong the Lew.1A skin-graft survival. With the H-1 plus non-H-1 antigenic difference (Fig. 3), skin grafts in two of eight animals had significantly prolonged survival times; the nonspecific effect of the serum was not at all apparent. In this group of animals, the possible presence of cytotoxic H-1 antibodies was also investigated. Serum samples were collected 17 d after skin grafting for the

**Fig. 2** Survival of Lew.1A skin grafts after treatment with Lew serum (●) AVN serum (○), untreated controls (△).**Fig. 3** Survival of Lew skin grafts after treatment with Lew serum (■), AVN serum (□), untreated controls (▲).

cytotoxicity test<sup>8</sup>. In comparison with the untreated controls (Table 1, Group 1) and Group 2 given syngeneic AVN serum, the Group 3 animals treated with Lew serum showed complete inhibition of anti-Lew antibody production.

In spite of the presumably low content of antigens in the serum, permanent tolerance of skin grafts can be induced in rats, at least in the non-H-1 incompatible combination. The antigenic difference between the donor and recipient strains is relatively strong, as shown by the acute rejection of skin grafts in untreated controls. With the H-1 plus non-H-1 difference, the degree of tolerance achieved is considerably lower. This does not, however, exclude the possibility that it can be increased by concentrated antigen from the serum. The nonspecific immunosuppressive effect of normal serum on non-H-1 incompatible skin grafts is consistent with previous findings<sup>9-14</sup>. To summarise the present data, it seems that the serum may be a very good source of soluble H antigens useful for the induction of transplantation tolerance.

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## Antibody response induced *in vitro* to the cell-surface alloantigen Thy-1

MANY important antibody responses are provoked by antigens on the surfaces of nucleated cells and, in spite of wide interest, little is known of the immunobiology of these responses. Alloantigens comprise an intriguing class of surface antigens because they may stimulate antibody production by B cells as well as target-cell killing by T cells and also because alloantigens are themselves intimately involved in the immunological recognition of other cellular antigens, such as viral antigens<sup>1</sup>, autoantigens<sup>2</sup>, surface-bound haptens<sup>3</sup>, other alloantigens<sup>4</sup> and tumour-associated antigens<sup>5</sup>. Studies of antibody responses *in vitro*, involving potent xenoantigens, have contributed greatly to the understanding of cellular and molecular interactions in immunity<sup>6</sup>. This has suggested the potential of such systems for the study of responses to weaker antigens of nucleated cells. In this report I define such an experimental approach with the induction *in vitro* of primary and secondary responses to the murine alloantigen Thy-1 as measured with a modified plaque-forming cell (PFC) assay, developed from the method of Fuji *et al.*<sup>7</sup>.

Thy-1 is present as two allelic variants, Thy-1.1 (strain AKR) and Thy-1.2 (CBA)<sup>8</sup>, and is expressed in high density at the surface of thymic lymphocytes which serve as target cells in the PFC assay. In initial experiments the induction of a response to Thy-1 was attempted by coculturing normal or primed AKR spleen cells with antigen in the form of viable CBA thymocytes. Very few PFC lytic to CBA thymus cells appeared although the duration of culture and cell ratios were varied. By contrast, the use of a cell-free supernatant of thymocyte cultures induced substantial PFC responses. A comparison of the ability of thymus cells and supernatant to induce a primary response is shown in Table 1. CBA but not AKR thymocyte supernatants (AKR-T-SN) induced the production of PFC from AKR spleen cells, at an optimal dilution of antigen of 1/15. Addition of CBA thymocytes to spleen cell cultures failed to generate PFC although they were present in numbers con-

sonant on the concentrations of cells which yield immunogenic supernatants ( $2.5 \times 10^6$ – $5.0 \times 10^6$  ml<sup>-1</sup>). In the absence of antigen, no PFC are detected in primary responses. Figure 1 illustrates the specificity of the primary response and also follows the response kinetics which show a maximum at 4 d. Later peak responses (5–6 d) have been found in other experiments. The AKR-T-SN, which fails to stimulate AKR spleen cells, can stimulate large anti-AKR PFC responses of CBA spleen cells (optimal dilution 1/10–1/25, data not shown). Reciprocity of the response, however, does not prove immunological specificity since it is possible that allogeneic supernatants simply induce polyclonal PFC responses. Further evidence for specificity was obtained through the use of third-party thymus supernatants derived from the congenic strains A/Thy-1.1 and A/Thy-1.2. Titrations of the immunogenicity of A/Thy-1.1-T-SN and A/Thy-1.2-T-SN paralleled precisely those of AKR-T-SN and CBA-T-SN, respectively (unpublished results). Since both A/Thy-1 strains have numerous alloantigens absent in AKR which excepting Thy-1.2, fail to induce PFC, it follows that the induction of PFC is antigen specific and attributable fully and exclusively to the Thy-1 antigen.

Secondary *in vitro* responses of AKR spleen cells from mice immunised *in vivo* with CBA thymocytes differ from primary responses in kinetics and in optimal antigen dose. The secondary response exhibits a sharp peak at 3 d and the optimal concentration of supernatant antigen is about 1/10 of that optimal for generating primary responses; an example of a secondary response is shown in Table 2. In this experiment the specificity of induction is evident, as AKR-T-SN produced only few PFC above a small background now detected in the absence of added antigen.

Although an antigenic difference at the Thy-1 locus induces the PFC response and other alloantigens fail to do so, it was possible that Thy-1 substance acted nonspecifically to augment immunity to another alloantigen or autoantigen and that the PFC response was not directed at Thy-1. This was examined by testing one pool of AKR anti-CBA PFC against a panel of thymus cells from strains AKR, A/Thy-1.1, CBA and A/Thy-1.2. Mean PFC per group on day 3 was 0, 0, 143, and 134, respectively, which rules out the possibility of autoantibody

**Table 1** Comparison of the ability of thymus cells and thymus-cell culture supernatant to induce a primary antibody response to Thy-1 *in vitro*

| Thymus cells as antigen | PFC per culture ( $\pm 1$ s.e.m.) (CBA target cells) | Culture medium as antigen (T-SN) | PFC per culture ( $\pm 1$ s.e.m.) (CBA target cells) |
|-------------------------|------------------------------------------------------|----------------------------------|------------------------------------------------------|
| CBA $50.0 \times 10^5$  | $3 \pm 2.3$                                          | CBA 1:5                          | $35 \pm 16.6$                                        |
| CBA $10.0 \times 10^5$  | $1 \pm 1.0$                                          | CBA 1:15                         | $164 \pm 11.0$                                       |
| CBA $5.0 \times 10^5$   | $2 \pm 1.2$                                          | CBA 1:75                         | $84 \pm 11.1$                                        |
| CBA $2.5 \times 10^5$   | $1 \pm 0.3$                                          | CBA 1:150                        | $50 \pm 22.3$                                        |
| CBA $1.0 \times 10^5$   | $0 \pm 0$                                            |                                  |                                                      |
| CBA $0.5 \times 10^5$   | $1 \pm 0.7$                                          | Medium alone                     | 0                                                    |
| AKR $50.0 \times 10^5$  | $0 \pm 0.3$                                          | AKR 1:5                          | $4 \pm 3.3$                                          |
| AKR $5.0 \times 10^5$   | $4 \pm 2.3$                                          | AKR 1:15                         | $10 \pm 2.0$                                         |
| AKR $1.0 \times 10^5$   | $1 \pm 1.0$                                          | AKR 1:75                         | $2 \pm 0.3$                                          |
|                         |                                                      | AKR 1:150                        | $3 \pm 1.3$                                          |

A modified Marbrook culture system was used<sup>9</sup> in which  $15 \times 10^6$  spleen cells, in 1 ml of appropriate medium were placed into the inner dialysis compartment and 15 ml of Eagle's modified minimal essential medium (MEM) containing 5% newborn calf serum (MEM-CA<sub>5</sub>) and 2-mercaptoethanol ( $5 \times 10^{-5}$  M) were added to the outer flask. Thymocyte culture supernatant was prepared by cultivating thymocytes ( $62.5 \times 10^6$  ml<sup>-1</sup>) in Dulbecco's modified MEM (E4) containing 10% foetal calf serum (E4-F<sub>10</sub>) for 20 h at 37 °C in 10% CO<sub>2</sub> and air and collecting the supernatants by centrifugation at 250g and passage through a Millipore membrane (0.22  $\mu$ m). PFC assays were based on the method of Fuji *et al.*<sup>7</sup>, and modified to accommodate a Trypan blue staining routine<sup>11</sup>. Briefly, aspirated cultures were collected into pellets by centrifugation at 100g for 15 min and 0.05 ml of a 12% suspension of appropriate thymocytes (in MEM-CA<sub>10</sub>) was added. The tubes were then warmed to 37 °C to receive 0.35 ml of 0.6% agar in E4-CA<sub>10</sub>, the latter maintained at 45–50 °C. After mixing, the suspension was applied to the surface of a 50-mm Petri dish containing 1–2 ml of solid 1% Agarose (Indubiose A37, L'Industrie Biologique Francaise, Gennevilliers) in phosphate-buffered saline (PBS). After gelation, 3.0 ml of E4-CA<sub>10</sub> was added to each of the dishes which were then incubated at 37 °C for 3.5 to 4.5 h. Each dish then received 1.5 ml of complement (rabbit serum absorbed with mouse spleen cells) diluted 1:15 in E4-CA<sub>10</sub>. After further incubation for 0.75 h the dishes were drained and stained with 1.5 ml of 0.2% Trypan blue for 20 min at 20 °C. The dishes were rinsed with PBS and the plaques were counted using a dissecting microscope (Baush and Lomb,  $\times 10$ ) adjusted for diffuse indirect illumination. In these experiments normal AKR spleen cells were suspended in 1 ml of various dilutions of thymocyte supernatant in E4-F<sub>10</sub> or in E4-F<sub>10</sub> containing various numbers of thymocytes, which were then added to the inner culture chamber. Triplicate or quadruplicate cultures were assayed for PFC after incubation for 4 d at 37 °C in a humid 10% CO<sub>2</sub>–90% air atmosphere.



**Table 2** Specificity of the induction of a secondary anti-Thy-1 response *in vitro* of AKR spleen cells by antigenic supernatants of thymocytes

| AKR supernatant antigen (AKR-T-SN) | PFC per culture ( $\pm 1$ s.e.m.) | CBA supernatant antigen (CBA-T-SN) | PFC per culture ( $\pm 1$ s.e.m.) |
|------------------------------------|-----------------------------------|------------------------------------|-----------------------------------|
| 1:25                               | 33 $\pm$ 19.7                     | 1:50                               | 198 $\pm$ 9.5                     |
| 1:125                              | (1) 21 $\pm$ 7.0                  | 1:125                              | (1) 340 $\pm$ 60.0                |
| 1:500                              | 17 $\pm$ 9.6                      | 1:500                              | 166 $\pm$ 61.9                    |
|                                    |                                   | 1:1,500                            | 55 $\pm$ 8.7                      |
| Medium alone                       | 12 $\pm$ 5.2                      | 1:5,000                            | 7 $\pm$ 3.7                       |

Cultures were assayed after 3 d for PFC. For priming, AKR mice received  $4 \times 10^7$  CBA thymus cells intravenously 2–8 weeks before use. The data were obtained with CBA target cells in the PFC assay; figures in parentheses are the mean autoantibody PFC per culture detected with AKR thymus cells as targets using portions of the same cultures.

production and shows that virtually all the *in vitro* secondary alloantibody PFC produce anti-Thy-1 antibody. Primary response PFC, however, do contain an interesting subpopulation (10–20%) of autoantibody PFC.

These experiments demonstrate that alloantibody responses to a surface antigen of nucleated cells can be induced entirely *in vitro*. Both primary and secondary responses are produced, the latter with *in vivo* primed spleen cells, and are shown to differ in kinetics and in sensitivity to antigen. In contrast with *in vivo* PFC responses, which are induced on challenge with viable thymus cells (ref. 7 and my unpublished results), the *in vitro* responses are induced only with antigenic supernatants of cultured thymocytes. A rapid appearance of immunogen ( $\leq 4$  h) in culture supernatants was found in preliminary studies consistent with the release of Thy-1 antigen from thymocytes detected by Vitetta *et al.*<sup>9</sup>. Therefore, the failure of thymus cells to stimulate PFC cannot be ascribed to a late release of antigen and is the subject of a study in progress. Preliminary experiments involving gel filtration indicate that the cell-free immunogen is much larger than molecular Thy-1; it therefore

probably comprises Thy-1 in association with other surface molecules. The contribution of the latter to the immunogenicity of Thy-1 remains to be determined.

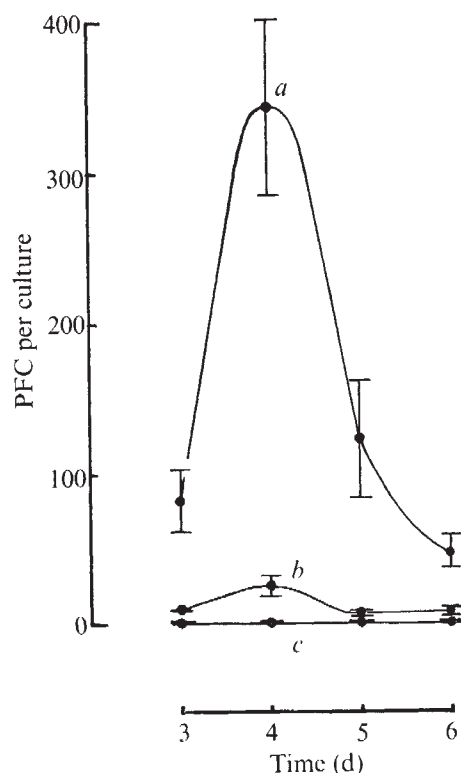
This experimental system, more generally, permits access to some basic problems of cell-surface immunogenicity, such as interactions among distinct surface molecules, the handling and presentation of antigen, the role of T cells and the determinants they recognise, control mechanisms of the response and the possibility of a rational approach to the manipulation of the response<sup>10</sup>. In this respect, cell-surface antigens in autoimmunity, and those of viral and tumour origin are interesting candidates.

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**Fig. 1** Kinetics and specificity of a primary anti-Thy-1 PFC response to CBA target cells induced *in vitro* with AKR spleen cells stimulated by thymus cell culture supernatant. *a*, Response to CBA supernatant diluted 1:25; *b*, response to AKR supernatant diluted 1:25; *c*, response to medium alone. Vertical bars represent  $\pm 1$  s.e.m.



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## Transition probability and the hormonal and density-dependent regulation of cell proliferation

WE have recently been studying cultured cells derived from the androgen-responsive mouse mammary tumour, Shionogi 115 (S115)<sup>1,2</sup>. Here we show, from time-lapse cinemicrographic analysis of these cells, that regulation of proliferation rates by both cell population density and physiological concentrations of testosterone is achieved by changes in 'transition probability'.

It is increasingly apparent that cell division depends on the occurrence of a random transition in  $G_1$  (refs 3–5). On this basis, a model of the cell cycle has been proposed which also quantitatively accounts for the observed heterogeneity of cell cycle times within cell populations<sup>3</sup>. According to this model, the cell cycle is divided into two parts; one called the B phase where cells are proceeding towards division, and the other called the A state, where cells are not proceeding to division but are 'waiting' for a random triggering event to occur before proceeding. A cell may remain in this A state for any length of time and it has been shown that in steady-state conditions, the cell

has a constant probability per unit time ('transition probability') of leaving the A state and entering the B phase<sup>3</sup>. It is thought that cells enter the A state in the G<sub>1</sub> phase of the cell cycle because the kinetics of entry of cells into the S phase are identical with the kinetics of entry into division<sup>6</sup>. The B phase then comprises part of G<sub>1</sub>, and S, G<sub>2</sub> and M phases of the 'classical' cell cycle. The A state has much in common with the conventional G<sub>0</sub> in that cells in 'A' or 'G<sub>0</sub>' are not proceeding towards division. Although G<sub>0</sub> is often regarded as a kind of refuge into which a fraction of cells can retreat when conditions are unfavourable for proliferation, all cells are supposed to enter the A state in every cell cycle, irrespective of their proliferation rate. Even optimally growing transformed fibroblasts enter the A state and then have a given transition probability of leaving and progressing to DNA synthesis and division.

According to the model, proliferation rates could be changed by altering either the duration of B phase ( $T_B$ ) or the transition probability, or both. There is evidence that proliferation rates of animal cells are regulated in the main by changes in transition probability ( $P$ ) rather than by changes in  $T_B$  (refs 4 and 5).

S115 cells are particularly interesting because physiological concentrations of testosterone have two distinct, reversible effects on the cells. First, at any density, testosterone stimulates the proliferation rate approximately twofold. Second, cells seeded in the presence of testosterone grow exponentially and fail to exhibit density-dependent regulation of proliferation rate until high densities ( $0.3 \times 10^6$  cells  $\text{cm}^{-2}$ ) are reached, whereas cells seeded in the absence of the hormone proliferate in a density-regulated manner; that is, proliferation rate decreases continuously over a wide range of increasing densities. Thus the phenomenon of density-dependent regulation of proliferation rate may be studied by comparing the behaviour of cells grown either in the presence or absence of a known, purified hormone.

Generation times of S115 cells growing in modified Eagle's medium have been analysed by time-lapse cinematography. The data is plotted as the logarithm of the percentage of undivided cells against age (Fig. 1). This type of curve is an  $\alpha$  curve, (where  $\alpha_t$  is the percentage of cells with intermitotic times  $\geq t$ )<sup>3</sup>. In an ideal population of cells having identical

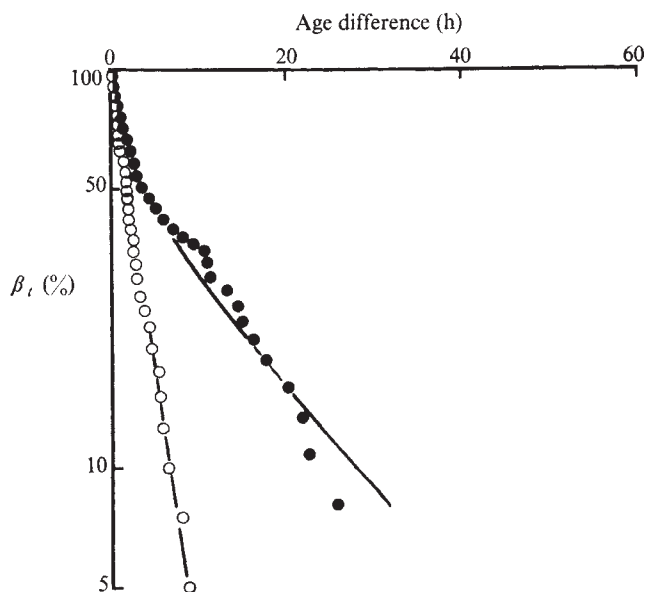
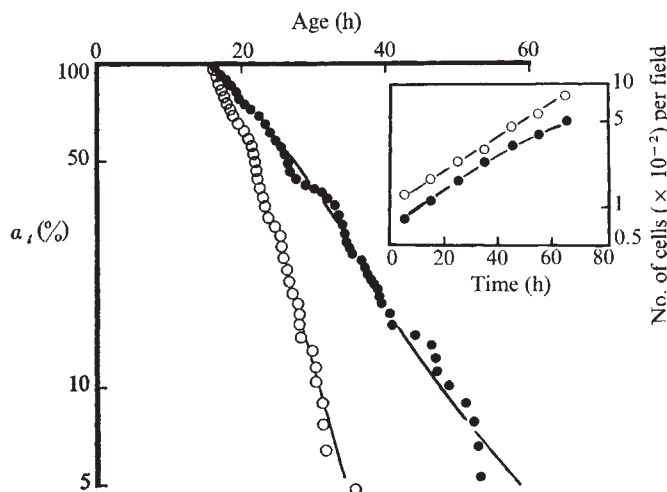


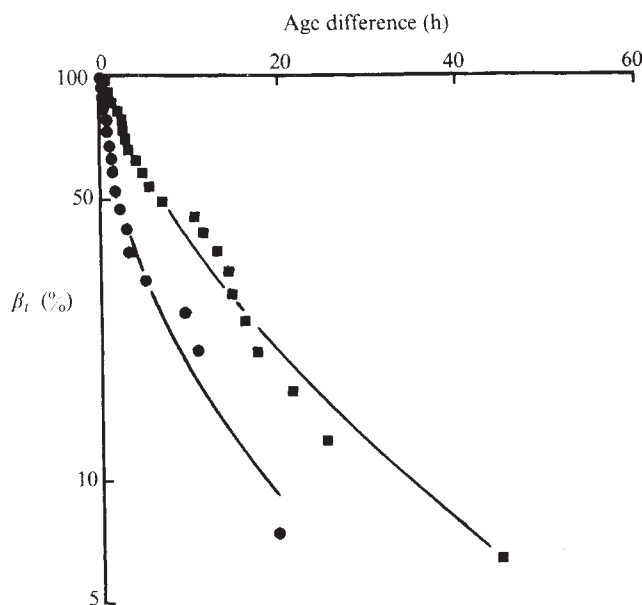
Fig. 2 Semi-logarithmic plot of % of sibling pairs with differences in intermitotic time  $> t$  ( $\beta$ ) against  $t$  for S115 cells. Data were taken from the same film as Fig. 1. Number of sibling pairs = 40 for +T; 43 for -T. Symbols as Fig. 1.

durations of B phases ( $T_B$ ) and identical transition probability,  $\alpha_t$  remains at 100% for ages up to  $T_B$ , then declines exponentially at a rate determined by the transition probability. It has been shown<sup>3</sup> that in steady-state cultures  $\alpha$  curves exhibit initial curvature, produced by variability in the length of the B phase ( $T_B$ ), followed by an exponential tail. The shortest intermitotic time gives the value of the minimum  $T_B$ , whereas the point at which the  $\alpha$  curve becomes linear gives the maximum  $T_B$ . If a given treatment were to change  $T_B$  and not  $P$ , then the  $\alpha$  curves for the treated and untreated populations would be parallel but laterally displaced from each other along the abscissa. Conversely, a change in  $P$  and not  $T_B$  would produce two lines with different slopes, but the same origin on the abscissa. It can be seen in Fig. 1 that the origins of the two curves for testosterone-treated and untreated cells are similar and the slopes of the exponential tails of the  $\alpha$  curves are different, indicating that the minimum  $T_B$  is unchanged, but transition probability is different in each case.

Calculation of transition probability from the  $\alpha$  curve is subject to some error since it is difficult to detect exactly when the initial curvature finishes. It has been shown that the differences in sibling intermitotic times can be used to give a better estimate of transition probability<sup>4</sup> because, in steady-state cultures, differences in intermitotic times of sibling cells are due entirely to differences in the times they spend in A state, their B phases being of identical duration<sup>7</sup>. The data in Fig. 2 are plotted on semi-logarithmic axes, giving a so-called  $\beta$  curve (where  $\beta_t$  is the percentage of sibling pairs with differences  $\geq t$ )<sup>7</sup>. Theoretically,  $\beta$  curves should yield a straight line plot if cells are growing exponentially, but not otherwise. Indeed this was found to be the case; the  $\beta$  curve for the exponentially growing, testosterone-treated cells gave a straight line, and that for the untreated cells curved upwards. The curvature indicates that transition probability is variable in the untreated cultures. To test whether this variability was a function of density, the data for sibling differences of the untreated cells were split into two groups on the basis of the time of division of the parent cell (Fig. 3). One group represents early divisions of the parent cell (less than 15 h after the beginning of the time-lapse film), whereas the other group represents later divisions (more than 15 h). The data in Fig. 3 show that the  $\beta$  curve of sibling cells dividing at later times is less steep than that of cells dividing at early times; that is, the mean transition pro-

Fig. 1 Semi-logarithmic plot of % cells undivided at age  $t$  ( $\alpha$ ) against  $t$  for S115 cells. S115 cells were cultured in Dulbecco's modified Eagle's medium containing 2% foetal calf serum and 30 mM HEPES as described previously<sup>6</sup>. Testosterone (T), when added, was present at  $3 \times 10^{-8}$  M. Exposures were made at 3-min intervals and individual intermitotic times measured by frame-by-frame analysis. The time of mitosis was taken when the cell rounded up before division. Number of observations = 93 for +T, 91 for -T.  $\circ$ , + testosterone,  $\bullet$ , -testosterone. Inset, increase in cell number during the course of the filming.





**Fig. 3** Semi-logarithmic plot of  $\beta$  against  $t$  for two groups of the untreated cells. ●, Analysis of siblings derived from parent cells which divided in the time interval 0–15 h from the beginning of the film. ■, Analysis of siblings derived from parent cells which divided at times > 15 h. Number of sibling pairs in the first group = 19; in the second group = 24.

bability of later dividers is less than that of early dividers. Furthermore, both curves still display marked curvature, indicating that transition probability decreases continuously with density. In addition,  $\alpha$  curves of the two groups demonstrated that there was little, if any, change in  $T_B$  with density (not shown).

In conclusion, both testosterone and cell population density affect proliferation rate by changing transition probability; both have little effect on the B phase of the cell cycle. The transition probability model may be successfully applied to non-steady-state cultures as well as to cultures growing exponentially.

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## Identification of the cell multiplication inhibitory factors in interferon preparations as interferons

THERE has been some controversy for the past few years concerning the inhibitory effect of interferon on cell multiplication. We and others have presented evidence that interferon itself is the responsible factor<sup>1–13</sup>. Some investigators have, however, failed to observe any inhibition of

cell multiplication with interferon preparations<sup>14–17</sup>, while others have observed inhibition but ascribed this effect to substances other than interferon in their preparations, either because no inhibition was observed when more purified preparations were used<sup>18</sup> or because of apparently inconstant ratios between antiviral and cell multiplication inhibitory (CMI) activities in a given preparation<sup>19–21</sup>. It has been claimed that the factor responsible for the CMI activity of human leukocyte interferon preparations could be physically separated from the factor responsible for the antiviral activity<sup>22</sup>. We provide strong evidence that the antiviral and CMI activities of interferon preparations are indeed intrinsic properties of the covalent structure of interferons.

Human<sup>23</sup> and mouse<sup>24</sup> interferon preparations can be reactivated after being unfolded under denaturing conditions in hot sodium dodecyl sulphate (SDS), a procedure which dissociates all non-covalently linked molecules<sup>25</sup>. The finding that the antiviral activity of interferon can be recovered after such treatment has enabled the separation and identification of distinct molecular forms of both mouse<sup>26,27</sup> and human<sup>28</sup> interferons by electrophoresis in SDS–polyacrylamide gels. Accordingly, in our experiments human leukocyte and mouse C-243 cell culture interferon preparations were boiled in the presence of SDS, either in non-reducing or reducing conditions, were subjected to electrophoresis in SDS–polyacrylamide gels, and gel fractions tested for antiviral and CMI activities.

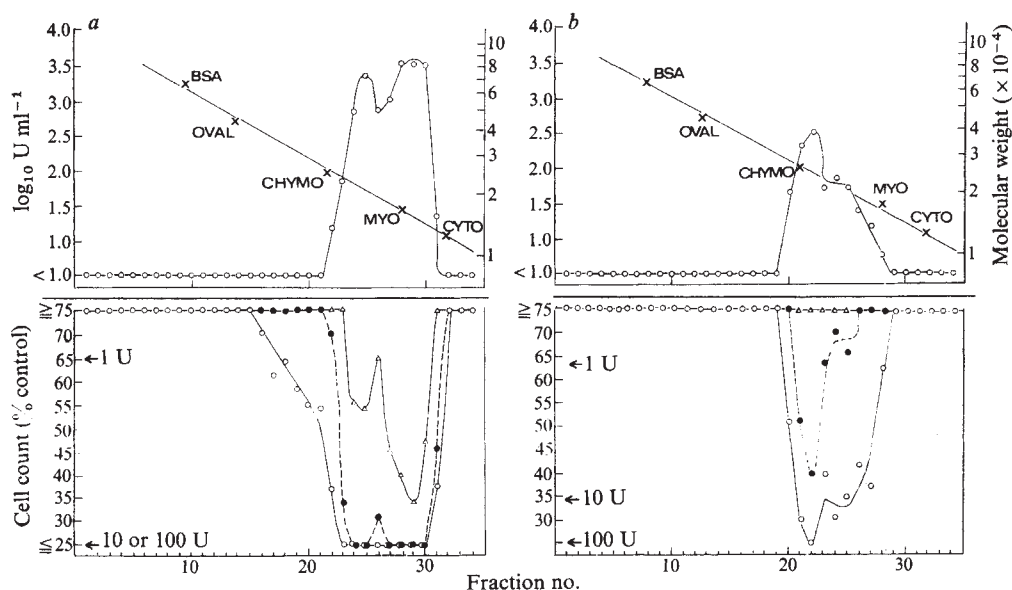
The human leukocyte interferon preparation, electrophoresed after boiling in SDS in non-reducing conditions, contained two peaks of antiviral activity, one at about 21,000 daltons and one at approximately 15,000 daltons (ref. 28 and Fig. 1a). The two peaks of antiviral activity coincide with the distribution of the CMI activity; there is a very good quantitative correlation between the antiviral activity and the CMI activity of each of the gel fractions.

When human leukocyte interferon was boiled in the presence of mercaptoethanol there was a loss of antiviral activity of the 15,000-dalton component, with only partial loss of the antiviral activity of the 21,000-dalton component (ref. 28 and Fig. 1b). Likewise, CMI activity was present in the 21,000-dalton component and markedly decreased or absent in the 15,000-dalton component (Fig. 1b).

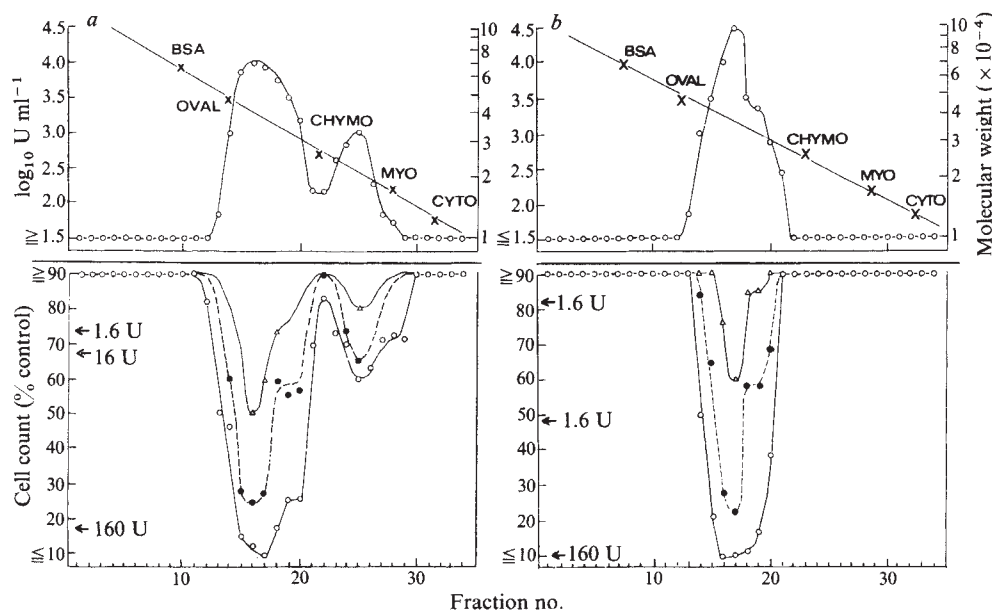
Unreduced mouse C-243 cell interferon exhibited a major peak of antiviral activity at an apparent molecular weight of 38,000 and a minor peak at about 22,000 (Fig. 2a). Thus, these mouse interferon preparations are similar in molecular heterogeneities to mouse interferon preparations produced in L-929 cells induced with Newcastle disease virus<sup>26</sup> and in L-pa cells induced with MM virus<sup>27</sup>. There was a direct quantitative correlation between the antiviral and CMI activities in the regions of the two peaks (Fig. 2a). Also when C-243 cell interferon was boiled in reducing conditions and electrophoresed in SDS–polyacrylamide gels, there was again a direct quantitative correlation between the two activities (Fig. 2b) and neither activity was present at 22,000 daltons.

In other experiments, gel eluates were assayed for both antiviral and CMI activities in cultures of L<sub>10-10</sub> cells, which were as sensitive as L-929 cells to the antiviral activity of mouse interferons and were approximately as sensitive as the L-1210 cells to the CMI activity of mouse interferon preparations. As in the experiments just described, the profiles of the two activities could be virtually superposed. Also, it was found that about 1% of both the antiviral and CMI activities of human leukocyte interferon crossed on mouse L<sub>10-10</sub> cells. When these mouse cells were used to assay gel eluates of electrophoresed human leukocyte interferon preparations, activity profiles were obtained for both antiviral and CMI activities which correlated with those found in human cell assays, with each fraction being active in the mouse cells at about 1% of its activity in human cells.





**Fig. 1** SDS-polyacrylamide gel electrophoresis of human leukocyte interferon assayed for antiviral and CMI activities in non-reducing (*a*) and reducing (*b*) conditions. *a*, Human leukocyte interferon induced by Sendai virus<sup>29</sup> containing approximately  $10^8$  U (human standard reference 69/19) and 2.5 mg protein per ml in 0.01 M phosphate buffer, pH 7.2 was constituted to contain 1% SDS and 5 M urea and was boiled for 1 min. An aliquot of 0.1 ml was applied to the gel and, following electrophoresis, 5-mm gel fractions were eluted in 2 ml Eagle's MEM containing 10% calf serum. Conditions for electrophoresis were as described previously<sup>26</sup>. Upper panel: antiviral activity was assayed on human diploid skin fibroblasts challenged with vesicular stomatitis virus (VSV). BSA, bovine serum albumin; OVA, ovalbumin; CHYMO, chymotrypsinogen; MYO, myoglobin; CYTO, cytochrome *c*. Lower panel: CMI activity was assayed on Burkitt lymphoma cells (Daudi line)<sup>30</sup>. All gel eluates were incubated with  $5 \times 10^4$  Daudi cells per ml in 2 ml RPMI medium containing 10% foetal calf serum, and cell multiplication was determined after 3 d incubation by counting viable cells in a haemocytometer using the Trypan blue exclusion test. Native human leukocyte interferon at 1, 10 and 100 U ml<sup>-1</sup> was used as reference. All eluates were tested at a 1:10 dilution (○) and, where indicated, at a 1:100 (●) or a 1:1,000 dilution (△). All points are averages of duplicate determinations. *b*, The same human leukocyte interferon preparation was constituted to contain 1% SDS, 1% mercaptoethanol and 5 M urea and was boiled for 1 min before application to gel.



**Fig. 2** SDS-polyacrylamide gel electrophoresis of mouse C-243 cell interferon assayed for antiviral and CMI activities in non-reducing (*a*) and reducing (*b*) conditions. *a*, Mouse C-243 cell interferon induced by Newcastle disease virus<sup>31</sup> containing approximately  $3 \times 10^6$  U and 1 mg protein per ml in 0.01 M phosphate, pH 7.2, was constituted to contain 1% SDS and 5 M urea and was boiled for 1 min. An aliquot of 0.1 ml was applied to the gel and, following electrophoresis, fractions were eluted as in Fig. 1. Upper panel: antiviral activity was assayed on mouse L cells challenged with VSV<sup>31</sup>. Lower panel: CMI activity was assayed on mouse leukaemia L-1210 cells<sup>3</sup>. All gel eluates were incubated with  $5 \times 10^4$  L1210 cells ml<sup>-1</sup> in 2 ml RPMI medium containing 5% horse serum, and cell multiplication was determined after 3 d incubation by counting viable cells in a haemocytometer using the Trypan blue exclusion test. Native mouse interferon at 1.6, 16, and 160 U ml<sup>-1</sup> was used as reference. All eluates were tested at a 1:10 dilution (○) and, where indicated, at a 1:100 dilution (●) or at a 1:1,000 dilution (△). All points are averages of duplicate determinations. *b*, The same mouse C-243 cell interferon preparation was treated in the same reducing conditions as in Fig. 1*b*, before application to gel.

The direct quantitative correlation between the antiviral and CMI activities of both human leukocyte and mouse C-243 cell interferons—(after boiling these interferons in SDS and subjecting them to electrophoresis in SDS-polyacrylamide gels)—shows that the CMI activity is exerted by molecules with heterogeneities of the same size as those of the interferons in the preparations. Furthermore, as there were two peaks of activity for un-reduced human leukocyte (21,000 and 15,000 daltons) and mouse interferon (38,000 and 22,000) the CMI factor is present in exactly the same proportion as the interferons of each of those sizes. Therefore, either both activities reside in the covalent structure of the same molecules (interferons), or there are two distinct populations of molecules that induce antiviral activity and two distinct populations of molecules, of the same two sizes and present in the same relative proportions as the two interferon populations, that induce CMI activity. Furthermore, in the case of human leukocyte interferon, each of these populations responsible for CMI activity, like the interferons, exert only ~ 1% of homologous activity in heterologous cells. The finding that the correlation between the two activities is unchanged when these interferons are boiled in reducing conditions and electrophoresed in SDS, indicates not only that the two activities reside in physically similar molecules (in terms of size heterogeneities) and are present in the same proportions, but that they are also due to chemically similar molecules (in terms of effects of reduction of disulphide bonds in detergent solution).

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## Antiviral and cell growth inhibitory activities reside in the same glycoprotein of human fibroblast interferon

It has been shown repeatedly that preparations of mouse interferon inhibit the growth of mouse cells *in vitro*<sup>1–4</sup>. In addition to inducing the antiviral state and inhibiting cell growth, preparations of mouse interferon stimulate interferon production by priming<sup>5</sup>, enhance phagocytic activity<sup>6</sup>, and potentiate in cells the toxic effect of double-stranded RNA<sup>7</sup>. All attempts with preparations of mouse interferon to separate the antiviral activity from the non-antiviral activities have been unsuccessful<sup>3,5,8</sup>. With human interferon few experiments have been reported, although the data available indicate inhibition of growth by preparations of human leukocyte interferon<sup>9–11</sup>. It has been claimed that the antiviral and cell growth inhibitory activities of human leukocyte interferon reside in different molecules and can be separated from each other by certain methods of fractionation<sup>12</sup>. In a more recent report<sup>13</sup>, however, separation of the two activities could not be demonstrated even in drastic denaturing conditions. I have shown that both the antiviral and the cell growth inhibitory activity of human fibroblast interferon reside in the same glycoprotein.

Human fibroblast interferon was purified to homogeneity as described previously<sup>14</sup>. Before the final fractionate step on sodium dodecyl sulphate (SDS)–polyacrylamide gel the interferon was heated at 100 °C for 2 min in a buffer of 0.1 M Tris, pH 6.8, 0.1% SDS, 1% β-mercaptoethanol, and 5 M urea. The interferon was homogeneous and identical both biologically and chemically to the homogeneous interferon described in ref. 14. This homogeneous interferon was used in all experiments. Interferon was assayed on human fibroblast cells by a microassay technique<sup>15</sup> with vesicular stomatitis virus (VSV) as the challenge virus. All interferon units are expressed as international units (human standard reference 69/19). Human diploid fibroblasts between the twentieth and thirtieth doubling obtained from neonatal foreskin tissue were seeded into plastic flasks (25 cm<sup>2</sup>) at 2 × 10<sup>4</sup>–8 × 10<sup>4</sup> cells per flask. Cells were grown at 37 °C in 4 ml of Eagle's MEM supplemented with 10% foetal calf serum, 1.1 g l<sup>-1</sup> sodium bicarbonate, 0.012 M HEPES buffer, 0.006 M Tricine buffer, and 50 μg ml<sup>-1</sup> gentomycin. The medium was changed every 5–7 d. In these conditions the cells grow with a doubling time of 35–40 h and to a final density between 1 × 10<sup>6</sup> and 2 × 10<sup>6</sup> cells per flask. The number of cells in each flask was counted in a haemocytometer after treatment of the cultures with a solution of 0.125% trypsin and 5 × 10<sup>4</sup> M versene in phosphate-buffered saline (PBS). Cell counts were performed every 24 or 48 h. Growth inhibition is defined as that inhibition which results in an increase of ≥ 50% in the time for cell doubling.

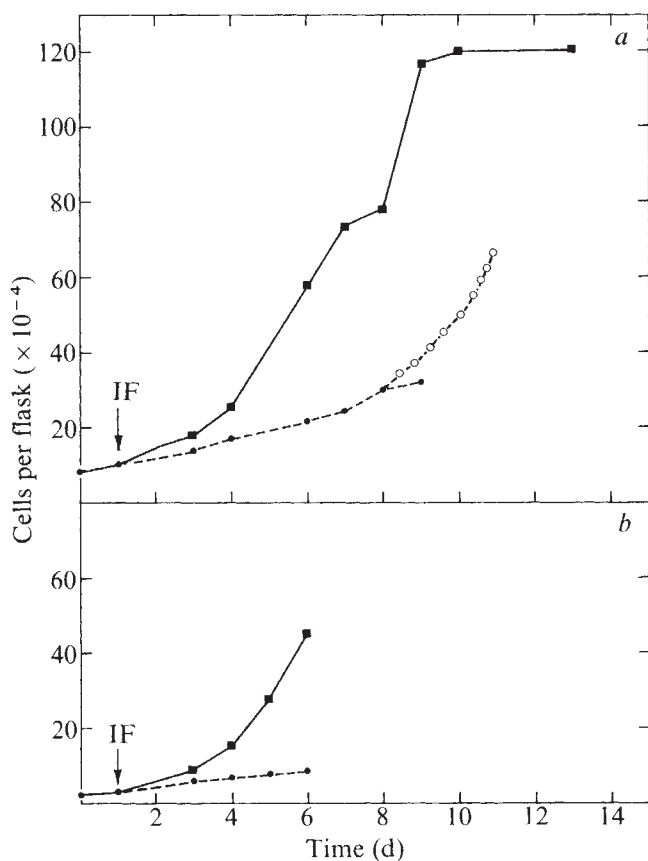
The effect of homogeneous human fibroblast interferon on the growth of fibroblast cells in culture is shown in Fig. 1. With either 200 U ml<sup>-1</sup> (Fig. 1a) or 500 U ml<sup>-1</sup> (Fig. 1b) the rate of cell growth is inhibited appreciably. The growth inhibition could be reversed by removal of the interferon-containing medium, washing the cells once with MEM then allowing the cells to grow in MEM only (Fig. 1a). Cells whose growth had been inhibited by the homogeneous interferon showed no toxic effects for the duration of the experiment (6–10 d, Fig. 1). Cells were routinely observed for 6–10 d after the addition of interferon. Caution

Another cultured cell, the Daudi cell line, derived from a pure preparations of interferon, for in an earlier exploratory experiment with a preparation of impure fibroblast interferon ( $1 \times 10^6$  U  $\text{mg}^{-1}$ ) a growth-inhibitory, but also toxic, effect was observed. In this case the cells rounded and detached from the surface 2–4 d after the addition of the interferon preparation (E. K., Jr, unpublished).

I conclude that the antiviral and growth inhibitory activity of human fibroblast interferon reside in the same glycoprotein. This may represent a difference between human fibroblast and leukocyte interferons since preparations of leukocyte interferon are reported to contain the two activities in separate molecules<sup>12</sup>. This separation of activities in preparations of leukocyte interferon has not been confirmed by electrophoresis of the interferon on polyacrylamide gel in conditions of drastic denaturation<sup>13</sup>. In the purification of the fibroblast interferon, growth inhibitory activity has not been found with the absence of the antiviral activity nor has a partial separation of the two activities been observed.

The very potent growth inhibitory activity of preparations of purified mouse interferon has been shown previously<sup>3</sup>. With the fibroblast cells, significant growth inhibition is observed at 200 U  $\text{ml}^{-1}$ . A molar concentration can therefore be calculated for growth inhibition as has been calculated for the antiviral activity of fibroblast interferon<sup>14</sup>. Thus, from an interferon concentration of 200 U  $\text{ml}^{-1}$ , a molecular weight of 20,000, (ref. 14) and a specific activity of  $2 \times 10^4$  U  $\text{mg}^{-1}$ , a concentration for a significant growth inhibition of human fibroblasts of  $4 \times 10^{-11}$  M is calculated.

**Fig. 1** Inhibition of the growth of human diploid fibroblast cells by homogeneous interferon. Cells were seeded at  $2 \times 10^4$ – $8 \times 10^4$  cells per flask in 4 ml medium. After 24 h the medium was changed in all flasks with half of the flasks getting medium containing interferon. *a*, Interferon, 200 U  $\text{ml}^{-1}$ ; *b*, interferon, 500 U  $\text{ml}^{-1}$ . ●, With interferon; ○, interferon removed; ■, control.



Another cultured cell, the Daudi cell line, derived from a Burkitt's lymphoma<sup>15</sup>, has been shown to be particularly sensitive to preparations of leukocyte interferon for growth inhibition<sup>17</sup>. The growth of Daudi cells can be significantly inhibited at 1–5 interferon units per ml (refs 13 and 17).

There is an extensive list<sup>18</sup> of polypeptide hormones which regulate cell growth both *in vivo* and *in vitro* but unlike interferon most of the polypeptide hormones stimulate cell growth. For instance, insulin stimulates DNA synthesis in resting 3T3 cells at  $3 \times 10^{-9}$  M<sup>19</sup>. Fibroblast growth factor shows measurable stimulation of DNA synthesis in 3T3 cells at  $4 \times 10^{-13}$  M and maximum stimulation at  $1 \times 10^{-10}$  M (ref. 20). Cell growth inhibition has been reported for adrenocorticotropin (ACTH)<sup>21</sup> and factors such as 'chalones'<sup>22</sup>.

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## Direct measurement of membrane motion and fluidity by electron microscopy

THE mobility of cell membrane components<sup>1</sup> has been measured either in the light microscope scale (by visual labelling<sup>2</sup> and by the recovery of fluorescent bleaching<sup>3,4</sup>) or at the molecular level (by nuclear magnetic resonance (NMR)<sup>5</sup> and spin label EPR<sup>6</sup> methods). In this report, a new method, using electron microscopic techniques to measure membrane motion, is described. This method reduces the spatial and temporal averaging processes inherent in many other methods, and extends the resolution limit of observation to the nanometer scale. The main difficulty in applying electron optical techniques to kinetic measurement had been the requirement that the specimen be placed in a vacuum. The recent development of environmental stages<sup>7</sup> has partly overcome this problem. The direct observation of reaction kinetics in an electron microscope is now possible. The application of this technique to biological research enables biological reactions to be observed at high resolution in a physiological environment.

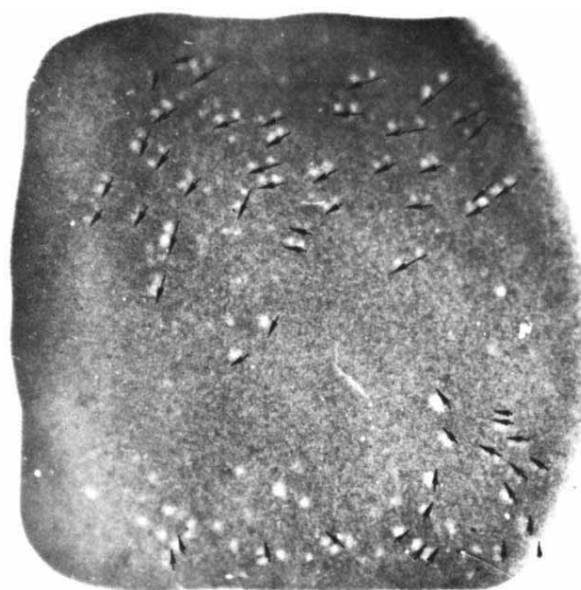
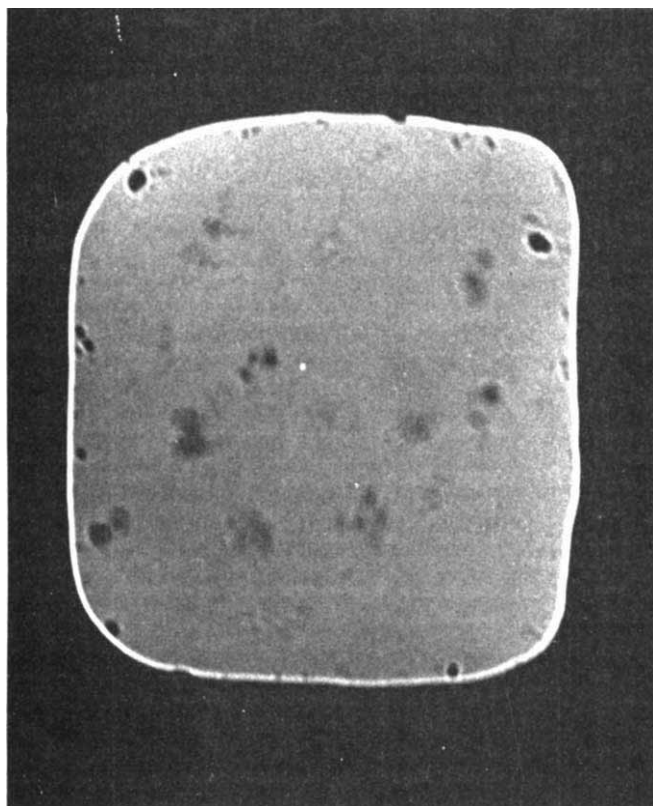
For model membrane studies, various molar mixtures of cholesterol, dipalmitoyl phosphatidylcholine and natural phosphatidylserine (extracted from bovine brain)<sup>8</sup> were used. The chloroform solution of the lipids were spread as monomolecular layers on the surface of the liquid subphase in a Langmuir trough. The subphase solution contained 5 mM Tris-HCl buffered to various pHs. Polystyrene latex spheres (Dow) of sizes 0.237  $\mu\text{m}$  or 0.088  $\mu\text{m}$  were introduced into the



subphase at a concentration of 0.005% solid. The charged latex spheres were electrostatically attached to the polar head group of the phospholipids at appropriate pH values. When bilayers were formed from the monolayers of the lipid mixtures as previously described<sup>9</sup>, the latex spheres adhered to the hydrophilic surface of the bilayers. They served as membrane markers when the unsupported single bilayer was viewed in an electron microscope in a hydration stage<sup>10</sup>. The marked bilayers were kept fully hydrated at all experimental temperatures. The electron beam current was kept below  $10^{-4}$  A cm<sup>-2</sup> at the specimen level, the beam being deflected from the area of interest between photographing periods. Electron micrographs were recorded on Kodak No-Screen X-ray film. The images of the unsupported bilayer membranes were extremely dim at the low beam current. Focusing was made on an area adjacent to the area of interest. Time-lapse micrographs of several second intervals, as well as timed exposures of several seconds, were taken.

Two types of particle motion were observed. The fast, random motion of the particles was best described as a two-dimensional Brownian motion. The loci of the particles consisted of zig-zag tracks of random walk, as shown in Fig. 1. The mean square displacements  $\bar{\Delta}^2$  were measured from the micrographs of exposure time  $\tau$ , and the two-dimensional lateral diffusion coefficients  $D$  of the membranes were deduced from Einstein's equation:  $\bar{\Delta}^2 = 4D\tau$ . For a bilayer of an equimolar mixture of dipalmitoyl phosphatidylcholine and cholesterol at 15 °C, the r.m.s. displacement of 20 labels was 0.54  $\mu$ m in 5 s. This value gave a diffusion coefficient  $D$  to be  $(1.4 \pm 0.3) \times 10^{-10}$  cm<sup>2</sup> s<sup>-1</sup>, the error being the standard deviation. This value is

**Fig. 1** Electron micrograph of the movement of 0.088- $\mu$ m polystyrene latex microspheres attached to an unsupported bilayer of an equimolar mixture of dipalmitoyl phosphatidylcholine and cholesterol at 15 °C in saturated water vapour. The exposure time is 5 s. The loci of the movement of the micrographs appear as blurred patches, with darkened spots indicating points of changing direction of the path of random walk. Bright fringes are caused by defocusing of the objective lens for contrast enhancement (magnification  $\times 7,480$ ).



**Fig. 2** The superposition of two negatives from consecutive electron micrographs of a microsphere-labelled bilayer of an equimolar mixture of dipalmitoyl phosphatidylcholine and cholesterol at 4 °C in saturated water vapour. The micrographs were taken 5 s apart. Arrows adjacent to the white sphere images indicate the relative positions of the microspheres from the first to the second micrograph (magnification  $\times 6,528$ ).

to be compared with those of bilayers of 4:1 molar mixtures of egg lecithin and cholesterol at 40 °C, that is  $1.9 \times 10^{-9}$  cm<sup>2</sup> s<sup>-1</sup> and  $1.5 \times 10^{-7}$  cm<sup>2</sup> s<sup>-1</sup> respectively from NMR<sup>5</sup> and spin label EPR<sup>6</sup> experiments. A recent measurement by fluorescent labelling method (W. W. Webb, personal communication) on egg lecithin and cholesterol bilayers also yielded a value of  $D$  in the range of  $10^{-8}$  cm<sup>2</sup> s<sup>-1</sup>. Our low value was probably a result of the large size of the labelling spheres, which were expected to cover thousands of lipid molecules. The motion of the labels represented the collective motion of many lipid molecules attached to the labels, instead of the diffusion of a single molecule. The grouping of lipid molecules by the labels was expected to impede the fast diffusion motion of individual molecules. The higher cholesterol content, saturated hydrocarbon chains and the lower temperature of our specimens, may also have accounted for some of the discrepancies. Detailed measurements of  $D$  as functions of temperature and composition by this method will be published elsewhere.

As the temperature of the specimen was lowered below the transition temperature of the bilayer membrane, the fast random motion was reduced. Time-lapse photographs of 10 s or more apart showed a convective movement of particles; the neighbouring particles often drifted along similar directions. Figure 2 shows the drift or marker particles. The mean drift velocity was  $3 \times 10^{-6}$  cm s<sup>-1</sup>. If this is taken to be the "diffuse" velocity, the apparent value of  $D$  would be  $10^{-11}$  cm<sup>2</sup> s<sup>-1</sup>, in rough agreement with NMR line shape measurement<sup>11</sup>. The coordinated drifting of particles indicated that the whole area moved as one patch. The boundaries between patches were discernible in the micrograph. The existence of patches or domains below the transition temperature agreed with the observations by electron microscopy<sup>12,13</sup> and diffraction<sup>9</sup>, and with much indirect evidence<sup>6</sup>.

Human erythrocyte membranes were prepared by the method of Jung<sup>14</sup>. The ghost membranes were labelled with latex microspheres of mean diameter 0.126  $\mu$ m. The binding of the charged spheres to the ghost membranes was checked by dark field light microscopy. The ghost membrane suspension was either placed on the Formvar-carbon coated grids or directly on uncoated fine mesh (1,500 CPI) grids. Excess liquid on

the grids was removed and the grids were viewed in the hydration stage. When uncoated grids were used, the labelled ghosts were seen to drift freely with the thin liquid film formed over the fine mesh holes. The movement of the marker particles in this case represented both the relative motion on the membrane, and the rotation and tumbling motions of the membranes as a whole. The latter type of motion was eliminated if the membrane sacs were deposited on Formvar-carbon substrates. An example of the marker movement on a ghost sac supported by Formvar-carbon substrate is shown in Fig. 3. The motion of the labels seemed to be more convective than diffusive even at 37 °C. (If the displacement of the labels was treated as diffusive, the apparent value of  $D$  would be in the order of  $10^{-11}$  cm<sup>2</sup> s<sup>-1</sup>.) A fluorimetric study<sup>15</sup> of the erythrocyte ghost membrane gave an upper limit of  $D$  to be  $3 \times 10^{-12}$  cm<sup>2</sup> s<sup>-1</sup>. Both results suggested that the diffusive motion in human erythrocyte ghost membrane was damped. It should be interesting to note that the apparent  $D$  values in 3T3 cell membranes, measured by a similar collective labelling method using a light microscope<sup>2</sup>, were the order of  $10^{-11}$  cm<sup>2</sup> s<sup>-1</sup>. A similar value was obtained from mouse L cell membranes by a fluorimetric study<sup>4</sup>.

By using an electron microscope, the size of the marker particles can be reduced below the resolution limit of light microscopes ( $\sim 0.5$   $\mu$ m), and the microscopic motion of the

membrane can be followed in greater detail. Smaller labels are now being tested. The membrane motion observed in the submicrometre scale is the result of the collective behaviour of membrane molecules, rather than the motion of the individual molecules. The detailed movement of the membrane in this scale has vital biological significance<sup>1</sup>. By studying membrane motion in this scale, one can bridge the knowledge obtained from measurements in the single molecular level<sup>5,6</sup> and that from bulk viscosity measurement<sup>2</sup>. Observation of movements of microdomains as well as specific sites on the cell surface such as anionic sites, antigens and lectin receptors may now be possible with specific labelling techniques.

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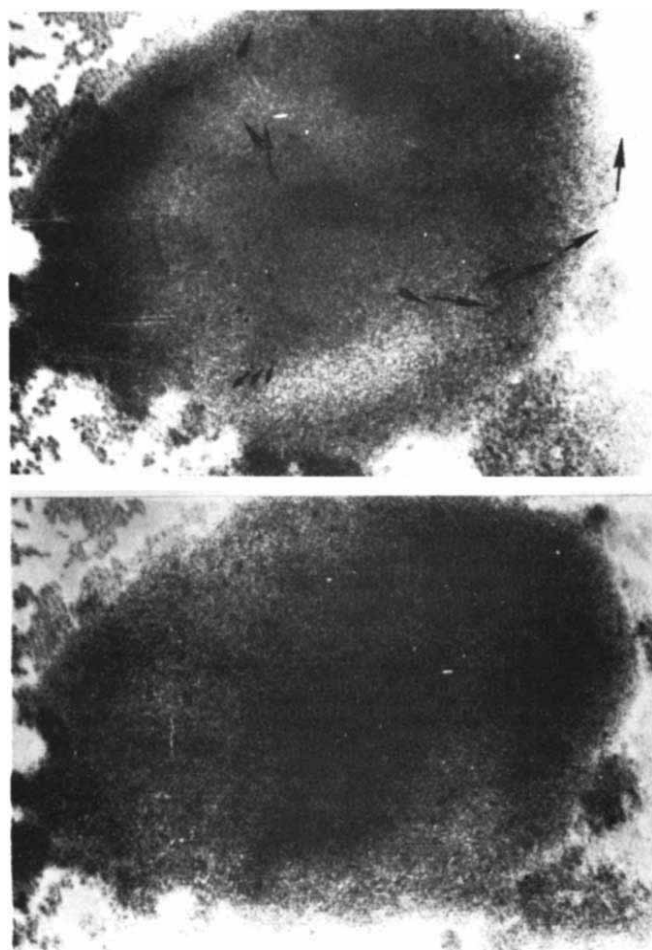
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Fig. 3 Electron micrographs of microsphere-labelled human erythrocyte ghost membrane taken 4 s apart. The membranes are kept fully hydrated at 37 °C. The arrows on the first micrograph indicate the direction of movement of the labelling microspheres to the positions on the second micrograph. The spheres appear as dark on the hydrated membrane sac. Large, dark patches are liquid (magnification  $\times 9,640$ ).



## Potential of central reward by localised perfusion of acetylcholine and 5-hydroxytryptamine

THERE is considerable evidence that the ascending catecholaminergic systems of the brain<sup>1</sup> are involved with behaviour that is contingent on reward. Much of this evidence concerns the variables which influence intracranial self-stimulation behaviour<sup>2</sup>. Because the anatomical and neurochemical correlates of self-stimulation are specified, evaluation of the catecholamine hypothesis should ideally involve the manipulation or measurement of neurochemical activity within restricted regions of the brain. We have made a first step in determining the neurochemical code of the excitatory input to the dopaminergic systems which originate in the ventral mesencephalon (VM) and which are thought to play a part in intracranial self-stimulation<sup>3</sup>. A technique well suited for this purpose is push-pull perfusion with which exogenous putative neurotransmitters can be administered to discrete regions of the brain by way of the perfusion medium. Using this technique it was possible to establish dose-response relationships between self-stimulation of the medial forebrain bundle (MFB), through which fibres of the catecholaminergic systems pass, and perfusion of 5-hydroxytryptamine (5-HT) or acetylcholine (ACh) in the VM.

In each of eight male Norwegian hooded rats (250–350 g) a monopolar stimulating electrode (diameter 0.25 mm) and a stainless steel guide cannula (20 gauge thin wall) were stereotactically implanted according to the coordinate system of De Groot<sup>4</sup>. The electrode was placed in the MFB (AP, 4.4; L, 1.7; HV, -9.0); and the guide cannula was



positioned so that its tip rested 2–4 mm above the sites intended for perfusion (AP, 3.0–2.0; L, 1.0–1.5; HV, –5.0).

Five to seven days after surgery the rats were taught to depress a lever in a Skinner box in order to receive intracranial electrical stimulation. The stimulation parameters were individually adjusted for each rat so that a rate of lever pressing approximately 50% of maximum was

obtained. Training continued until response rates were stable over a period of 90 min. Subsequently, that period was divided into three consecutive periods of 30 min which were designated pre-perfusion, perfusion and post-perfusion. The electrical stimulation parameters were identical for each of these three periods. The average frequency of lever pressing for the pre- and post-perfusion periods was determined. The frequency of lever pressing for the perfusion period was then expressed as a percentage deviation from this value.

Because push-pull systems available at present often cause excessive traumatization of brain tissues<sup>7</sup> a modified system was developed for use in the present study. Briefly, this system comprises the addition of a calibrated monitor tube to the pull side of the normally closed push-pull system. The level of fluid in this tube indicates the pressure in the pull line and gives immediate notice of any obstructions occurring within the brain. This facility prevents the inadvertent induction of expansion lesions and experimental hydrocephalus.

At the start of the "perfusion period" a concentric push-pull cannula assembly<sup>8</sup> was lowered through the guide cannula until its tip extended 2–4 mm beyond that of the guide. The rate of perfusion was between 0.6 and 1.7  $\mu\text{l min}^{-1}$ . The perfusion was continued during the second experimental period and was continuously monitored for blockages occurring within the pull line. In the event of a blockage the cannulae were temporarily removed, flushed clear and replaced.

The perfusion medium was an artificial cerebrospinal fluid (CSF) consisting of: NaCl, 127.7 mM; KCl, 2.5 mM; CaCl<sub>2</sub>, 1.3 mM; MgCl<sub>2</sub>, 0.9 mM; NaHCO<sub>3</sub>, 21.0 mM; Na<sub>2</sub>HPO<sub>4</sub>, 2.5 mM and glucose 3.4 mM. To this stock solution the required amounts of 5-hydroxytryptamine, creatine sulphate or acetylcholine chloride were added immediately before each experiment.

Each of six animals was tested with four doses of ACh, while each of four animals was tested with four doses of 5HT. The four dose levels, as well as a CSF vehicle control condition for both ACh and 5HT, were administered in a different random order for each animal. Successive perfusions with a given animal were separated by at least 24 h.

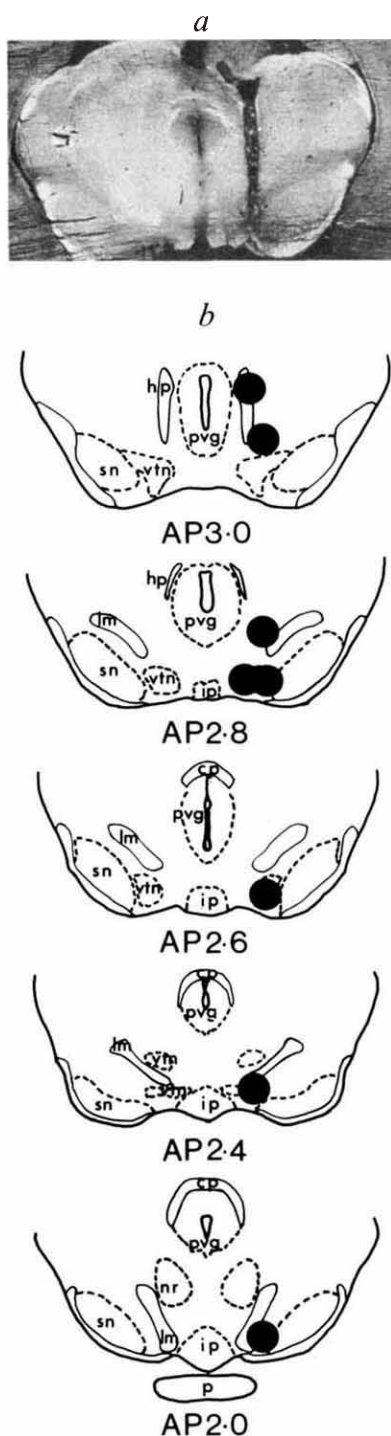
After completion of all behavioural testing, the position of the electrode tip and the site of perfusion in each animal were verified according to normal histological procedures. Brain tissue was fixed by an intracardial perfusion of a 10% formal saline solution after which it was embedded in paraffin wax. Serial sections of 8  $\mu\text{m}$  were cut and stained with haematoxylin using the modified Weil-Weigert method of Wolf<sup>7</sup>.

All electrodes were found to be located in the MFB region within 0.5 mm of the stated stereotaxic coordinates. Fig. 1a shows the type of lesion which is produced by the present push-pull perfusion technique, and Fig. 1b represents the loci of all perfusion sites.

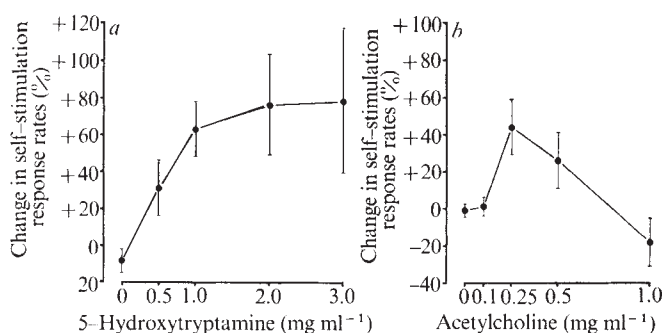
The mean percentage changes ( $\pm$  s.e.) in MFB self-stimulation of four rats, when different doses of 5-HT were perfused in local areas of the VM, are shown in Fig. 2a. 5-HT increased self-stimulation in each of the four rats, an effect which was statistically significant ( $P < 0.02$ , Kruskal-Wallis one-way analysis of variance<sup>9</sup>) for the group as a whole. In two of the animals the facilitatory effect of 5-HT disappeared at the two highest doses of 5-HT. Also in two animals, the effects of 5-HT on self-stimulation were accompanied by marked contraversive turning behaviour.

Figure 2b illustrates the mean percentage changes ( $\pm$  s.e.) in MFB self-stimulation of six rats when different doses of ACh were perfused in the VM. Following a significant increase in self-stimulation behaviour ( $P < 0.02$ , Kruskal-Wallis one-way analysis of variance) response rates were depressed below baseline levels as the concentration of ACh in the perfusate was increased. Facilitation of self-stimula-

**Fig. 1 a**, A histological section of rat brain showing a lesion made by the push-pull cannulae. The section was obtained from the brain of one of the animals used in this study. **b**, Representative frontal sections of rat brain modified from the stereotaxic atlas of Pellegrino and Cushman<sup>3</sup> indicating the confirmed placement of perfusion sites. cp, Posterior commissure; hp, fasciculus retroflexus; ip, interpeduncular nucleus; lm, medial lemniscus; nr, red nucleus; p, pons; pvg, central grey substance; sn, substantia nigra; vtn, Tsai's ventral tegmental nucleus.







**Fig. 2** *a*, Illustration of the mean percentage changes ( $\pm$  s.e.) in MFB self-stimulation of four rats when different doses of 5-HT were perfused in the ventral mesencephalon. *b*, Illustration of the mean percentage changes ( $\pm$  s.e.) in MFB self-stimulation of six rats when different doses of ACh were perfused in the ventral mesencephalon.

tion by ACh was observed in each of the six animals at the dose of 0.25 mg ml<sup>-1</sup>.

The present results show that a given animal can be perfused repeatedly with the modified push-pull system without causing excessive physical trauma to the brain. The push-pull perfusion was used in preference to the intracranial microinjection technique for two reasons. First, the push-pull perfusion provides a degree of temporal control over chemical stimulation of the brain which is impossible to replicate with microinjections. Secondly, the continual replacement of fresh ACh from the push syringe obviates the need for substitute cholinergic agonists or cholinesterase inhibitors.

The finding that MFB self-stimulation was potentiated by localised perfusions of 5-HT in the VM is contrary to the inhibitory influence of 5-HT on self-stimulation suggested by Wise *et al.*<sup>9</sup> and by Poschel *et al.*<sup>10</sup>. The position adopted by these authors was based on experiments which involved the administration of pharmacological or neurotoxic agents either systemically or by way of the cerebral ventricles. These procedures will affect neurochemical activity in large areas of the brain. In contrast, the present results were obtained by perfusing 5-HT within restricted regions of the VM. The differences between the general and specific manipulation of 5-HT systems suggests an anatomical coding of brain function in which 5-HT may be used at different morphological locations in order to achieve opposite or competing behavioural or physiological effects.

The favoured explanation of the present results is that 5-HT and ACh may be exciting, directly or indirectly, the dopaminergic cell bodies known to be present in the VM. Supporting this view is, first, the fact that in some animals, marked contraversive rotation was observed in response to 5-HT. Dopaminergic systems in the VM have been strongly implicated in mediation of this behaviour<sup>11</sup>. Second, a pathway originating in the serotonergic cell group of the median raphe, from which self-stimulation can be obtained, terminates in the ventral mesencephalic tegmentum<sup>12</sup>. Third, self-stimulation of the serotonergic cell bodies of the median raphe can be modified by drugs which affect mainly catecholaminergic systems of the brain<sup>13</sup>. This implies that activity initiated in the median raphe might be relayed postsynaptically by way of catecholaminergic systems. The results of the present experiments suggest a possible anatomical substrate for the proposed interaction between serotonergic and catecholaminergic systems involved in central reward. Fourth, acetylcholinesterase has been localised within the dopamine-containing neurones of substantia nigra<sup>14</sup>, presumably this is to inactivate ACh released from neurones afferent to the substantia nigra.

Although the proposed explanation of the present results may be satisfactory for those perfusion sites located in or near areas containing the dopaminergic cell bodies<sup>1</sup>, further investigation will be needed to clarify the role of 5-HT and ACh at the more dorsally located perfusion sites.

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## Stimulation of cerebral histamine H<sub>2</sub> receptors by clonidine

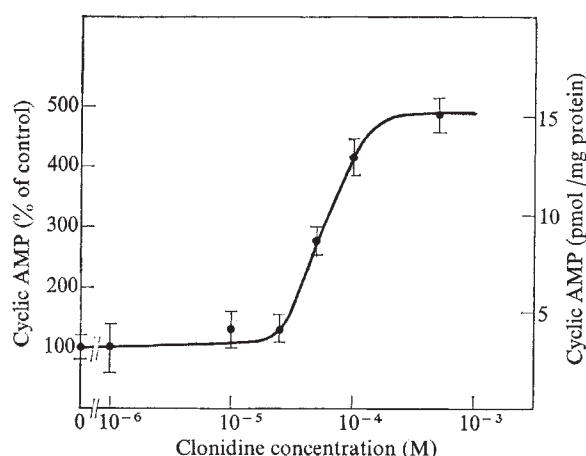
CLONIDINE (2-[(2,6-dichlorophenyl)amino]-2-imidazoline) is a potent antihypertensive agent whose site of action is believed to be in the central nervous system<sup>1</sup> but its mechanism of action is far from clear. Because of its effects on peripheral tissue, it has been generally accepted that the drug stimulates alpha-adrenergic receptors<sup>1</sup>. A recent report has, however, challenged this view: far from stimulating the receptors responsible for the accumulation of cyclic AMP in rat cortex, the drug seems to act as a potent alpha-adrenergic antagonist<sup>2</sup>. On the other hand, clonidine has been reported to stimulate gastric secretion by activation of histamine H<sub>2</sub> receptors<sup>3</sup>. It was therefore of interest to check whether clonidine could also act on H<sub>2</sub> receptors in brain tissues where they have been characterised<sup>4-6</sup>. We now report that clonidine strongly stimulates the accumulation of cyclic AMP in slices from guinea pig brain through activation of H<sub>2</sub> receptors.

Male Hartley guinea pigs (300 g; Janvier, Le Genest) were killed by decapitation and slices were prepared from the hippocampus and incubated according to Baudry *et al.*<sup>4</sup>. The hippocampus was selected because of the strong stimulation of adenylate cyclase elicited by histamine in this region<sup>6</sup>. Cyclic AMP was assayed at the end of 15-min incubations by the protein kinase binding method and proteins were determined by the method of Lowry *et al.* with bovine serum albumin as standard<sup>4</sup>.

As shown in Fig. 1, clonidine induced a concentration-dependent accumulation of cyclic AMP. The half maximal stimulation was obtained with 50  $\mu$ M and the maximal effect, representing a fivefold increase, was achieved by 500  $\mu$ M.

The nature of the receptors involved in the effect of a supramaximal concentration of the drug was investigated with different kinds of antagonists.

Neither phentolamine, an alpha-adrenergic antagonist, nor timolol, a specific blocker of beta-receptors, nor mepyramine, a histamine H<sub>1</sub> antagonist, had any effect,



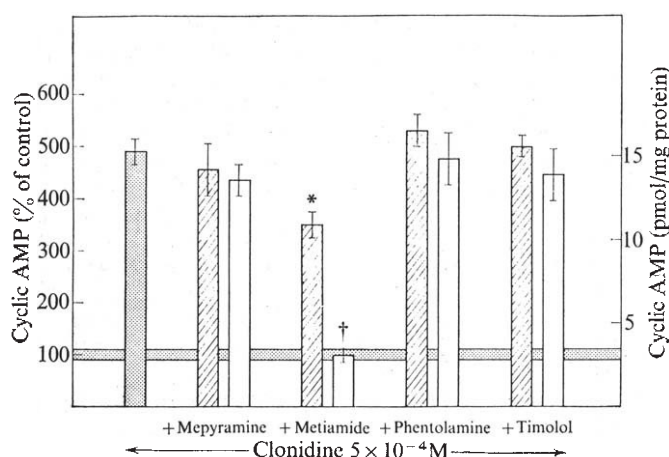
**Fig. 1** Effect of increasing concentrations of clonidine on cyclic AMP accumulation in slices from guinea pig hippocampus. Each value represents the mean  $\pm$  s.e.m. of three to four experiments.

even at 100  $\mu$ M, on the clonidine-induced stimulation. On the other hand, the action of clonidine was partially inhibited by 1  $\mu$ M of the  $H_2$  antagonist, metiamide<sup>7</sup> and totally blocked at 100  $\mu$ M of this agent. Furthermore, the stimulation elicited by 100  $\mu$ M clonidine and 100  $\mu$ M 4-methylhistamine, an agonist of  $H_2$  receptors were not additive (Y.A., A.V., and J.S., to be published).

Taken together these results clearly indicate that clonidine stimulates histamine  $H_2$  receptors in brain tissues, as already demonstrated in stomach<sup>3</sup> and heart<sup>8</sup>. In fact clonidine seems to be one of the most potent agonist of  $H_2$  receptors available: both the  $EC_{50}$  and the maximal stimulation of cyclic AMP accumulation were of the same magnitude as for 4-methylhistamine (Y.A., A.V., and J.S., to be published).

It is not clear whether this action of clonidine on cerebral histamine receptors is related to its hypotensive properties. Since this drug exerts its cardiovascular effects at very low dosage (in the range of 100  $\mu$ mol  $kg^{-1}$ ), its strong efficacy *in vivo* hardly seems compatible with the  $EC_{50}$  determined in our *in vitro* system. In this respect, the blockade of

**Fig. 2** Effect of antagonists of  $H_1$  or  $H_2$  histaminergic and alpha- or beta-adrenergic receptors on the accumulation of cyclic AMP elicited by a supramaximal concentration of clonidine. None of the agents affected the basal level of cyclic AMP. Each value is the mean  $\pm$  s.e.m. of four experiments. The width of the horizontal line represents the s.e.m. of unstimulated controls. Blank areas,  $10^{-4}$  M; hatched areas,  $10^{-6}$  M. \* $P < 0.005$ . † $P < 0.0005$ .



adrenergic alpha-receptors in slices from rat brain occurs at a much lower concentration<sup>2</sup>. In addition, very little information is available concerning the possible involvement of histaminergic neurones in the central control of blood pressure. It has only been demonstrated<sup>9</sup> that the intra-ventricular administration of histamine elicits tachycardia and hypertension in several species; however this action seems to be mediated by way of  $H_1$  receptors and the effects of a selective stimulation of cerebral  $H_2$  receptors remain to be assessed.

On the other hand, the high magnitude of the stimulation of the cyclic AMP system that we observe, together with the almost complete inhibition by intraventricular metiamide of the clonidine-induced hypotension which has been recently reported by Karppanen *et al.*<sup>10</sup> lend strong support to the idea that histamine  $H_2$  receptors in brain could be involved in the action of clonidine.

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## Synaptic vesicle cytochemistry changes when cultured sympathetic neurones develop cholinergic interactions

THE principal neurones of the rat superior cervical ganglion (SCG) can be established as dissociated cells in long term cultures<sup>1</sup> without the survival of the small intensely fluorescent "interneurone" normally found in the animal. These isolated sympathetic neurones (SCGN) exhibit certain expected adrenergic characteristics such as the synthesis and accumulation of catecholamines<sup>2-4</sup>. They also specifically take up exogenous noradrenaline and demonstrate  $Ca^{2+}$ -dependent release of this transmitter during induced depolarisation<sup>5</sup>. When these neurones are cocultured with thoracic spinal cord explants, they receive a typical cholinergic preganglionic input<sup>6</sup>. When cultured with the appropriate target of brown fat, the SCGN will provide characteristic noradrenergic contacts with the fat cells<sup>7</sup>. The interpretation of these generally expected results is confounded by the following observations: (1) cultures of dissociated sympathetic neurones form numerous synaptic contacts with each other<sup>8-10</sup>; (2) cytochemical studies of these synapses show that they contain predominantly dense-core vesicles and thus appear to be noradrenergic<sup>8-11</sup>; and (3) physiological studies clearly indicate that synaptic signals between these cultured neurones are nicotinic-cholinergic<sup>7,11</sup>. In addition, dissociated SCGN will provide cholinergic innervation to striated muscle in culture<sup>12</sup>. Only one type of neurone, however, has been described in these cultures. We have investigated whether synaptic endings with the morphological characteristics of noradrenergic terminals were the only synaptic type to be found in cultures

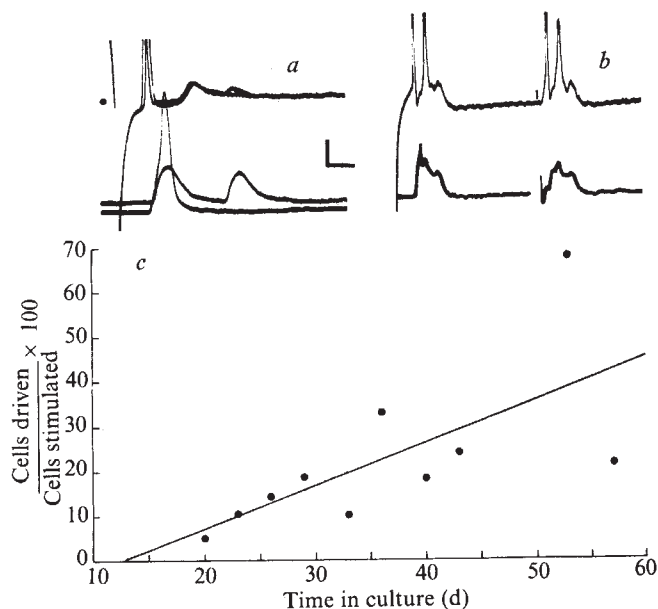


where cholinergic interactions between neurones were readily demonstrable. If, as previously reported, only synapses with noradrenergic cytochemical characteristics were found, it would strongly suggest that one synaptic type was handling both noradrenaline (NA) and acetylcholine (ACh). If two synaptic types were found it would suggest that two types of neurones were present in these cultured networks, thus providing a possible explanation for the paradoxical results discussed above.

We established sympathetic neuronal networks by dissociating the SCG from perinatal rats by a method previously described<sup>9</sup>, but without antimitotic agents to suppress non-neuronal cell growth. In these conditions a substantial number of non-neuronal cells are present although these do not develop to the point of forming a confluent monolayer. We chose these conditions that foster cholinergic mechanisms (discussion in ref. 13) although we have observed cholinergic transmission in cultures entirely free of supporting cells<sup>7</sup>. All results presented here derive from one group of 60 cultures that was established simultaneously from one group of foetuses and maintained in an identical manner. After initial settling on to 24-mm Aclar dishes, each culture contained about 2,000 neurones, a number which remained essentially constant throughout the experiment. The use of a gridded culture dish assisted in counting the number of neurones per dish. Less extensive studies of previous culture series confirmed the results. The methods used for anatomical, biochemical and physiological analyses are given in the figure legends.

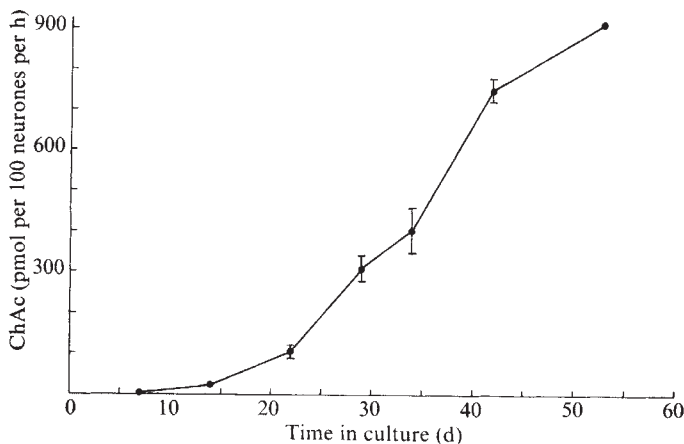
Choline acetyltransferase (ChAc) activity, which was very low in these cultures when first established, increased with age. ChAc activity in SCGN, expressed relative to number of neurones, increased more than 1,000-fold between one and 7.5 weeks (Fig. 1). The increase in ChAc activity during the development of SCGN *in vitro* probably reflects increased biosynthetic capacity for the production of ACh. The presence of this enzyme is considered a reliable indicator of the presence of cholinergic mechanisms<sup>14-16</sup>. During the same period acetylcholinesterase and dopa decarboxylase activities increased 30- and 20-fold, respectively, in this same culture series (our unpublished observations).

Physiological recordings indicated that nicotinic-cholinergic (hexamethonium-sensitive) synaptic interactions



**Fig. 2** Development of synaptic interactions as a function of days in culture. Intracellular recordings were obtained with 1M potassium citrate electrodes from SCGN that were maintained with a continuous perfusion of Eagle's MEM supplemented with antibiotics or with medium containing  $10^{-4}$  M hexamethonium<sup>6</sup>. Evoked synaptic potentials were sought by sequentially stimulating, with anode break excitation, each of a pair of simultaneously impaled neurones. Examples of recordings taken from a 33- and 53-d culture are shown in *a* and *b*, respectively. *a*, Two superimposed stimulus trials of the cell shown in the upper trace (L9) evoked short latency subthreshold or suprathreshold responses in the cell shown in the lower trace (R6). Electrical stimulation of R6 did not reveal reciprocal connections to L9. The first subthreshold potentials in L9 (upper trace) were due to recurrent connections; late potentials occurring concurrently in both neurones were due to spontaneous activation from some unknown source. *b*, Direct stimulation of cell shown in upper trace (L3) from a 53-d culture evoked a short latency abortive action potential, followed by multiple subthreshold potentials, in cell shown in lower trace (R3) as well as recurrent suprathreshold plus multiple subthreshold responses in L3. Stimulation of R3 (shown as break in lower baseline) evoked a reciprocal suprathreshold response in L3 which, in turn, elicited the same complex sequence of responses seen when L3 was stimulated electrically. Only single responses were seen in young cultures (20-33 d) whereas repetitive and suprathreshold activation was frequently observed in older cultures (36-57 d). In more than 25 pairs of cells studied pharmacologically, hexamethonium reversibly blocked the synaptic activity. Calibration: *a*, 10 mV, 20 ms and *b*, 10 mV, 50 ms. *c*, Plot of the ratio of cells synaptically driven to number of cells stimulated as a function of age in culture. Each dot based on recordings from an average of 50 cells (range 40-60) from a single culture. Positive slope of linear regression line ( $r = 0.66$  with  $P < 0.05$ ) suggests that the probability of finding synaptically coupled neurones increases with age in culture.

**Fig. 1** Increase in ChAc activity with time in culture. ChAc activity was assayed radiometrically at 37 °C in homogenates of cultures by a modification<sup>18</sup> of existing methods<sup>19,20</sup>. Enzyme activity for each culture was expressed relative to the number of neurones in that culture. Values for time points between 7 and 53 d are means  $\pm$  s.e.m. of activities in eight, eight, three, three, three, four and two cultures, respectively. The average number of neurones per culture was  $2,160 \pm 92$  ( $n = 31$ ). ChAc activities at 7 and 14 d were  $0.83 \pm 0.1$  and  $23.9 \pm 3.7$  pmol ACh produced per 100 neurones per h, respectively.



between the SCGN increased in potency and complexity with age in culture (Fig. 2*a* and *b*). In young cultures (20-33 d) only single responses (Fig. 2*a*), often with high failure rates, were observed. Reciprocal connections, frequent suprathreshold responses, and repetitive discharges (Fig. 2*b*) were first seen after 36 d in culture. The increased efficacy of synaptic transmission with age *in vitro* correlated with increased probability of finding interactions (Fig. 2*c*).

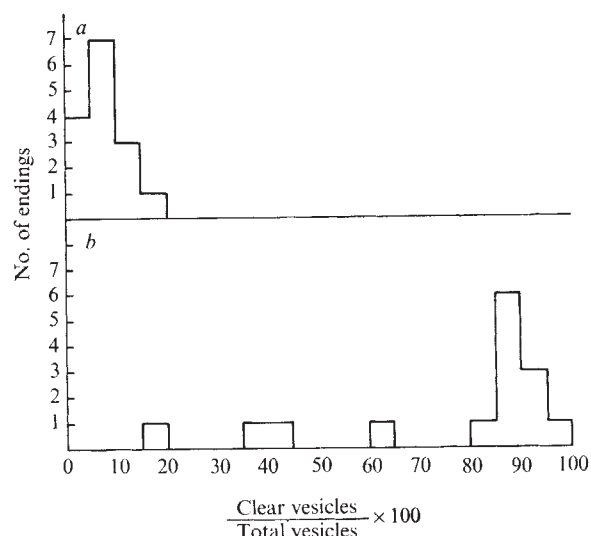
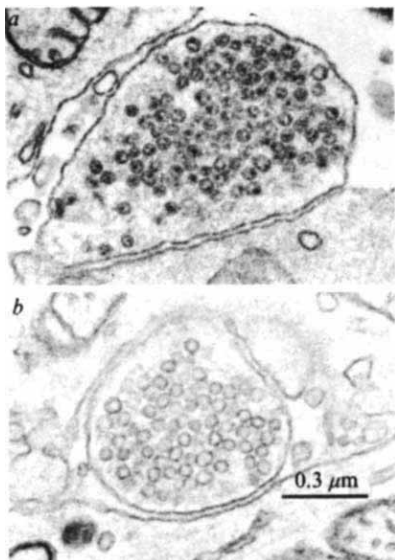
Detailed morphological studies were carried out on these SCGN cultures at 1 and 8 weeks. Preloading with NA and  $\text{KMnO}_4$  fixation techniques were used specifically to demonstrate catecholamine-containing dense-cored synaptic vesicles (see legend to Fig. 3). Figure 3 illustrates examples of synaptic profiles in contact with neuronal somata and proximal dendrites after 1 week (Fig. 3*a*) and 8 weeks (Fig. 3*b*). The vesicle population in the endings in the 1-week culture was predominantly dense-cored with few clear vesicles. In contrast, the synapse shown from the 8-week



culture contained mainly clear vesicles. Less than 10% of the population of synaptic vesicles was clear in most terminals examined after 1 week, whereas by 8 weeks most terminals contained more than 80% clear vesicles (Fig. 4). But some profiles after 8 weeks retained a high percentage of dense-cored vesicles (as in Fig. 3a) with a low proportion of clear vesicles. On one neuronal soma from an 8-week culture, eight synaptic profiles contained principally clear vesicles and one profile showed a predominance of vesicles containing dense cores. Occasionally at 8 weeks, endings containing a large number of clear vesicles had several vesicles with striking dense cores. Some profiles consisted of two to three connected varicosities each of which contained the same predominant type of synaptic vesicle. Studies in progress indicate that the shift in type within the synaptic vesicle population may occur as early as 2 weeks *in vitro*.

These results indicate that acetylcholine synthesis, cholinergic transmission and synaptic terminals containing clear vesicles develop with time in culture, a critical change occurring around 2 weeks *in vitro*. This development is gradual and not total since profiles containing principally dense-cored vesicles are still present at 8 weeks. As the number of neurones per culture dish remains stable after 1 week, these results suggest that one neurone type maintains two distinct synaptic endings and neurotransmitter enzyme systems or, alternatively, that two different neuronal types are present after 2 weeks *in vitro*. The critical question now centres on the source of the large numbers of cholinergic neurones present in older cultures. The small number (about 5%) of cholinergic neurones which may be present in the normal rat SCG<sup>17</sup> could not explain the accumulation of the large number of cholinergic

**Fig. 3** Electron micrographs of synaptic endings found on SCG neurones after 1 week (a) and 8 weeks (b) in culture. Dissociated neurones of the SCG dissected from perinatal rats were established in culture as described before<sup>1</sup>. Instead of a Leibovitz-based medium, a standard feed containing 65% Eagle's MEM with glutamine added, 25% human placental serum, 10% chick embryo extract, 3% 1.1 M glucose and nerve growth factor was used. At weekly intervals from 1 to 8 weeks the cultures were rinsed in Leibovitz medium (L-15) and incubated for 30 min in freshly prepared  $10^{-5}$  M noradrenaline in Leibovitz to which ascorbic acid ( $0.2 \text{ mg ml}^{-1}$ ) was added. The pH was adjusted to 7.2–7.3 before incubation. A rinse in L-15 was followed by fixation with 3%  $\text{KMnO}_4$  in Krebs' Ringer phosphate (pH 7.1,  $4^\circ\text{C}$ ) and by *en bloc* staining with 1% uranyl acetate in maleic acid buffer (pH 5.2,  $4^\circ\text{C}$ ). Embedding in Epon-Araldite followed dehydration. As expected with this fixation, postsynaptic densities are not demonstrated. Both (a) and (b) are at the same magnification.



**Fig. 4** Number of endings (ordinate) containing listed percentages of clear vesicles (abscissa) after 1(a) and 8(b) weeks *in vitro*. Consecutive endings found on the neuronal somata and proximal dendrites (as illustrated in Fig. 3) were photographed and printed at a standard magnification to count and classify vesicles measuring 40–70 nm as dense-cored or clear. Fifteen total endings at both 1 and 8 weeks are illustrated. Total vesicles ranged from 18 to 162 and 26 to 163, respectively.

“driver” cells found in late cultures (Fig. 2). Are these cells gradually evolving in culture from one originally undetermined multi-potential precursor population or from a differentiated noradrenergic neurone whose properties are altered in the culture environment? We anticipate that an extensive characterisation of the types of synaptic profiles present during successive weeks *in vitro*, together with additional biochemical and physiological experiments, will resolve this issue.

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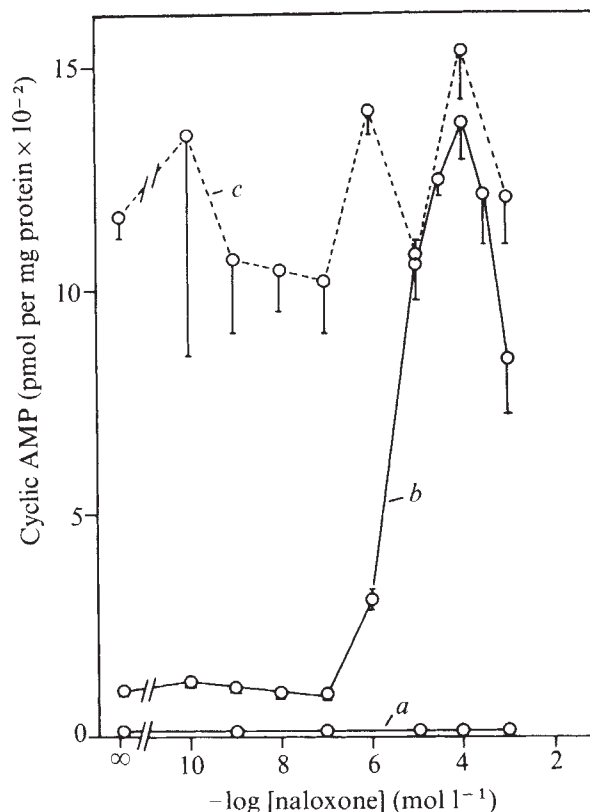
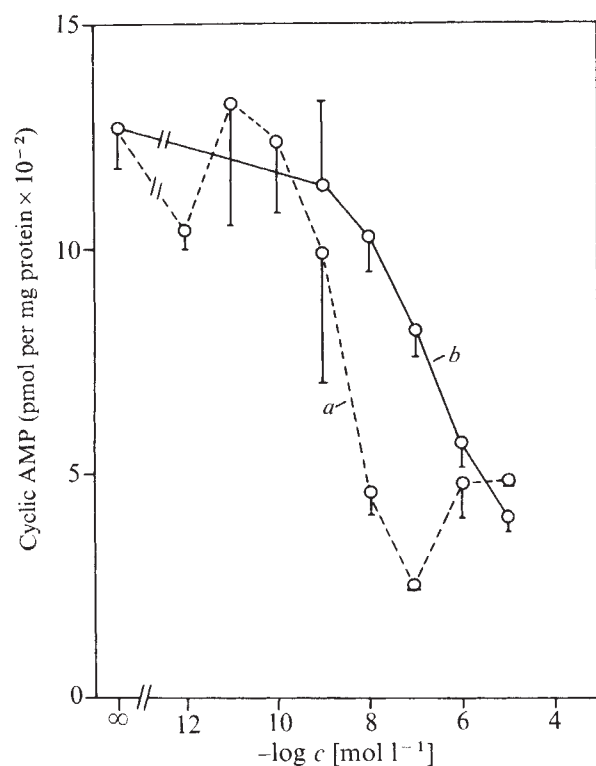
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## Enkephalin regulates the levels of cyclic nucleotides in neuroblastoma x glioma hybrid cells

NARCOTICS do not normally occur *in vivo*. "Opiate receptors" must therefore have evolved to recognise endogenous ligands, which are mimicked by the opiates<sup>1</sup>. Indeed such compounds have been isolated<sup>2-5</sup> and their structure elucidated as that of the two pentapeptides leucine- and methionine-enkephalin<sup>6</sup>. Neuroblastoma x glioma hybrid cells 108CC15 display many properties characteristic of neurones<sup>7</sup>. In these cells morphine and other narcotics<sup>8-10</sup> prevent the increase in the level of cyclic AMP that is caused by prostaglandin E<sub>1</sub> (PGE<sub>1</sub>)<sup>11</sup>. The opiates also increase the level of cyclic GMP in the hybrid cells<sup>12</sup>. On long term incubation with morphine, the hybrid cells show phenomena which can be interpreted as biochemical correlates of morphine tolerance, dependence and withdrawal<sup>9,13</sup>.

**Fig. 1** Leu-enk (*a*) and levorphanol (*b*) inhibit the elevation by PGE<sub>1</sub> (0.3  $\mu$ M) of the level of cyclic AMP in the neuroblastoma x glioma hybrid line 108CC15.  $2.5 \pm 10^5$  viable cells were seeded on to replica plates (85 mm in diameter) containing 20 ml of growth medium<sup>11</sup>. The cells were grown for 4 d before the medium was removed. The cells were first washed and then incubated with 5 ml of incubation medium<sup>14</sup> and the various additions at 37 °C for 10 min. Leu-enk was added as a 100-fold concentrated solution in water. 1.9 million viable cells per plate, 90% viability (exclusion of nigrosin), passage number 17. After the incubation the cellular content of cyclic AMP was determined as described<sup>14</sup>, with the exception that the columns for the purification of cyclic AMP were modified<sup>12</sup> and that the nucleotide was eluted with 5 ml of 0.05 M HCl. Each value is the mean  $\pm$  s.d. of data obtained from three parallel incubations. Leu-enk was synthesised by conventional methods coupling Z-Tyr-(BZL)-Gly-Gly-OH<sup>15</sup> and H-Phe-Leu-OH<sup>16</sup> by the mixed anhydride procedure to yield Z-Tyr-(BZL)-Gly-Gly-Phe-Leu-OH (melting point 181–183 °C ( $\alpha$ )<sub>646</sub><sup>20</sup> –25.8 ° and ( $\alpha$ )<sub>D</sub><sup>20</sup> –21.7 °, c, 1.06 in dimethylformamide). Hydrogenolytic removal of the protecting groups led to chromatographically and sequentially homogeneous leu-enk (melting point 197–199 °C (dec.); ( $\alpha$ )<sub>546</sub><sup>20</sup> +35.3 ° and ( $\alpha$ )<sub>D</sub><sup>20</sup> +29.1 °, c, 1.15 in water; amino acid ratios in acid hydrolysate: Tyr 1.00 (1), Gly 1.95 (2), Phe 1.00 (1), Leu 1.00 (1); ultraviolet maximum (water) 274.5 nm,  $\epsilon$  = 1,350 M<sup>-1</sup> cm<sup>-1</sup>).



**Fig. 2** Naloxone prevents the inhibition by leu-enk of the PGE<sub>1</sub>-induced increase in the level of cyclic AMP. *a*, Absence of PGE<sub>1</sub> and leu-enk; *b*, Presence of 0.3  $\mu$ M PGE<sub>1</sub> and 0.1  $\mu$ M leu-enk; *c*, Presence of PGE<sub>1</sub>.  $2.3 \times 10^5$  viable 108CC15 cells per plate, viability 92%, passage number 16. Other details as in Fig. 1.

Here we report that leucine-enkephalin (leu-enk) has effects like those of opiates on the hybrid cells.

PGE<sub>1</sub> strongly elevates the level of cyclic AMP in the hybrid cells<sup>11</sup>. Increasing concentrations of leu-enk progressively prevent this increase (Fig. 1*a*) until maximal inhibition is observed at 0.1  $\mu$ M (concentration at which 50% inhibition takes place, IC<sub>50</sub>, 3 nM). In the range 0.1–1  $\mu$ M leu-enk the curve rises again, but there is no further increase between 1 and 10  $\mu$ M leu-enk (Fig. 1*a*). In several independent experiments the same results were obtained. Such an increase in the level of cyclic AMP was also observed at concentrations above 0.1 mM of morphine and its congeners<sup>8</sup>. Levorphanol (Fig. 1*b*) is a less potent inhibitor of the PGE<sub>1</sub> elevation of cyclic AMP than leu-enk. Its IC<sub>50</sub> value (0.3  $\mu$ M), which is identical to that reported<sup>9</sup>, is approximately 2 orders of magnitude higher than that of leu-enk. In the absence of PGE<sub>1</sub> 1  $\mu$ M leu-enk maximally lowers the level of cyclic AMP from  $19.8 \pm 2.6$  to  $9.6 \pm 1.4$  pmol mg<sup>-1</sup> protein. As is the case with morphine<sup>10,17,18</sup>, the action of leu-enk is due to an inhibition of the formation of cyclic AMP and is not associated with an increase in cyclic AMP phosphodiesterase activity. The increase from  $796 \pm 176$  in the presence of PGE<sub>1</sub> to  $4,940 \pm 220$  seen in the presence of PGE<sub>1</sub> and the phosphodiesterase inhibitor isobutylmethylxanthine is partially suppressed to  $2,590 \pm 60$  pmol cyclic AMP per mg protein if 0.1  $\mu$ M leu-enk is included in the incubation medium.

Naloxone completely prevents the inhibition by leu-enk of the PGE<sub>1</sub>-induced rise in the level of cyclic AMP (Fig. 2*b*). In the absence of leu-enk (Fig. 2*c*), or of PGE<sub>1</sub> and leu-enk (Fig. 2*a*), naloxone has no effect.

As is the case with morphine<sup>12</sup> (Fig. 3*a*), leu-enk (Fig. 3*b*) causes an elevation of the intracellular level of cyclic GMP. The half-maximal stimulating concentrations (EC<sub>50</sub>) of morphine and leu-enk are around 0.1  $\mu$ M (Fig. 3*a*) and 0.3 nM

(Fig. 3b), respectively. From one experiment to another these  $EC_{50}$  values vary considerably, but they seem to change in parallel with one another.

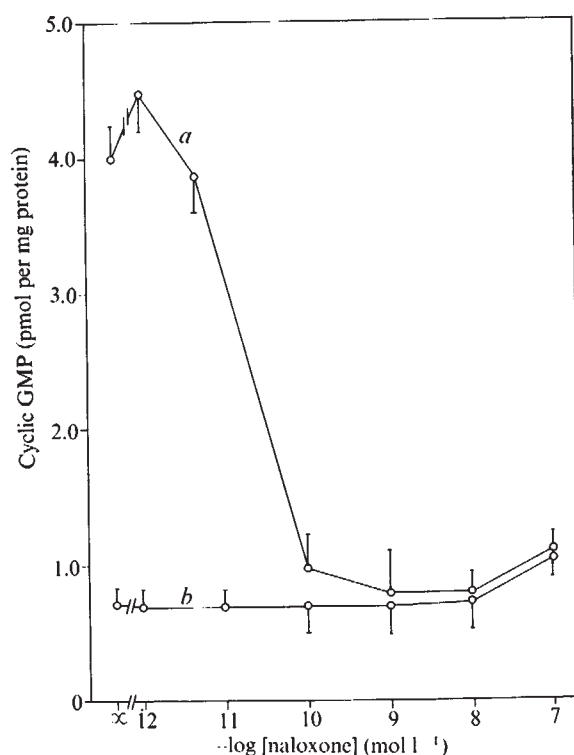
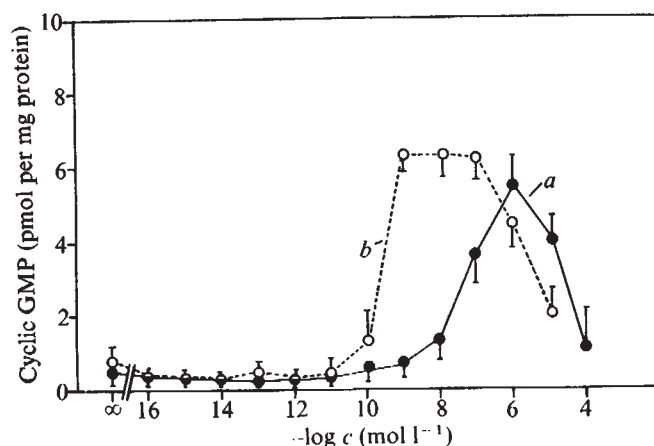
Naloxone prevents the increase in the level of cyclic GMP that is elicited by leu-enk (Fig. 4a). Its  $IC_{50}$  value is 20 pM. Up to a concentration of 10 nM, naloxone alone causes no effects of its own on the level of cyclic GMP. At more elevated concentrations it raises the level of cyclic GMP even in the absence of leu-enk (Fig. 4b).

The influence of leu-enk or opiates on the levels of cyclic nucleotides are strongly dependent on the presence of high concentrations (154 mM) of  $Na^+$ , as used in the present experiments. In the absence of  $Na^+$ , leu-enk at pM concentrations elevates the level of cyclic AMP (these authors, in preparation). This phenomenon may be related to the observation that opiate agonist binding is favoured at low, and antagonist binding at high concentrations of sodium ions<sup>19,20</sup>.

Besides the short-term effects of leu-enk, long term effects similar to those reported for morphine<sup>9,13</sup>, can be observed. If the hybrid cells are preincubated for 6 h with 1  $\mu$ M leu-enk, the level of cyclic AMP that is reached during the subsequent main incubation (10 min) with 0.3  $\mu$ M  $PGE_1$  rises from  $830 \pm 40$  to  $1,190 \pm 130$  pmol cyclic AMP per mg protein. If 1  $\mu$ M leu-enk is added at  $t = 0$  and 3 h of the 6 h preincubation period, the subsequent stimulation by  $PGE_1$  raises the level of cyclic AMP to  $1,830 \pm 140$  pmol per mg protein. Details of these experiments will be published elsewhere (our results, in preparation).

The data presented demonstrate an analogous influence on the hybrid cells of opiates and leu-enk. The opiates and leu-enk both depress the rate of formation of cyclic AMP and cause an increase in the level of cyclic GMP. The effects are all blocked by naloxone. Data almost identical to these for leu-enk were obtained with methionine-enkephalin. The similarity in the actions of opiates and leu-enk is paralleled by their structural similarity (J. R. Smythies, personal communication, and refs 21 and 22). The physiological meaning of the increased levels of cyclic GMP remains to be discovered. The short term action of opiates, that is, the depression of the formation of cyclic AMP, is probably correlated with its analgesic effect<sup>23,24</sup>. From analogy then, one may conclude that the enkephalins are neurohormones that, in the brain, suppress the sensation of pain. From studies on animals<sup>25</sup> and the neuroblastoma  $\times$  glioma hybrid cells 108CC15 (refs 9 and 13) it could be deduced that the phenomena of tolerance, withdrawal and dependence seen after long term influence of morphine are connected with increased formation of cyclic AMP. The fact that one does not become addicted to one's endogenous enkephalins is probably

**Fig. 3** Morphine (a) and leu-enk (b) elevate the levels of cyclic GMP in hybrid cells 108CC15.  $1.0 \times 10^6$  viable cells per plate, viability 95%, passage number 17. The cells were incubated in incubation medium for 30 s and their content of cyclic GMP was determined as described<sup>12</sup>.



**Fig. 4** Naloxone inhibits the increase in the level of cyclic GMP caused by leu-enk. a, Presence of 0.1  $\mu$ M leu-enk; b, absence of leu-enk.  $1.2 \times 10^6$  viable cells, viability 97%, passage number 14. Other details as in Fig. 3.

due to the degradation of the material<sup>13</sup>. In contrast, with the cell culture system, long term effects do occur. This extends the analogy between opiates and the enkephalins to their long term effects. It leads to the prediction that an inhibitor of the degradation of enkephalin, if applied properly to an animal, may cause opiate tolerance and dependence. The long term effects on the hybrid cells also suggest that breakdown of leu-enk in the culture does occur, although at a moderate rate.

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## Enkephalin and opiate narcotics increase cyclic GMP accumulation in slices of rat neostriatum

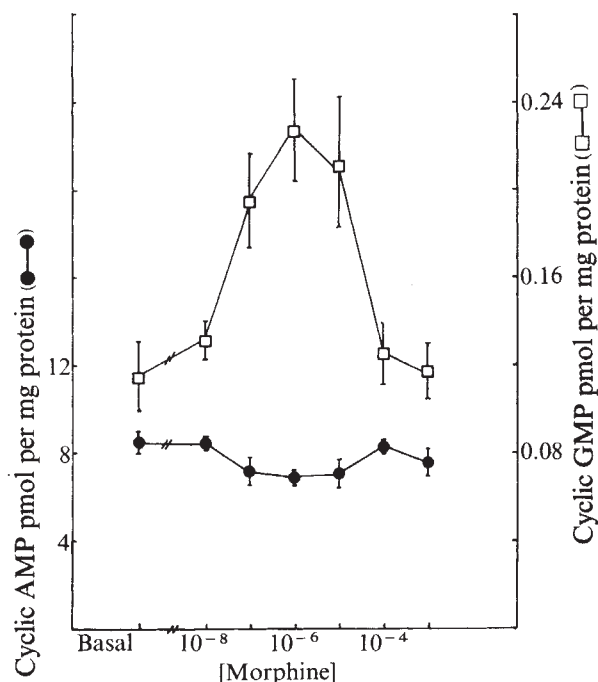
SEVERAL recent studies suggest that cyclic nucleotides are involved in the cellular actions of the opiate narcotics<sup>1–3</sup>. Following the observation by Collier and Roy<sup>1</sup> that low concentrations of morphine inhibited prostaglandin E<sub>1</sub> (PGE<sub>1</sub>)-stimulated adenylate cyclase activity in rat brain, Traber *et al.*<sup>2</sup> showed that morphine acted similarly to block the rise in intracellular 3',5'-adenosine monophosphate (cyclic AMP) induced by PGE<sub>1</sub> in cultured neuroblastoma, or in a hybrid cell line of neuroblastoma × glioma with a high density of opiate receptor binding sites. Sharma *et al.*<sup>3</sup> found that morphine inhibited basal adenylate cyclase activity in this cell line and Gullis *et al.*<sup>4</sup> observed that morphine increased the 3',5'-guanosine monophosphate (cyclic GMP) and reduced the cyclic AMP content of these cells. Racagni *et al.*<sup>5</sup> have also demonstrated that injection of analgesic doses of morphine *in vivo* to rats also increased cyclic GMP concentration in the neostriatum. These effects have generally been found to be stereospecific and blocked by the opiate receptor antagonist drug naloxone, suggesting that they are mediated through specific opiate receptors. We have extended these observations to *in vitro* studies on slices of rat neostriatum, an area with a very high density of opiate receptor binding sites<sup>6</sup>, and have also examined the effects of enkephalin on this system.

Slices of rat neostriatum were incubated for 15 min at 37 °C in Krebs–Ringer bicarbonate buffer containing 1 mM isobutylmethylxanthine (IBMX) (a potent phosphodiesterase inhibitor) and added drugs, as previously described by Forn *et al.*<sup>7</sup> (see legend to Fig. 1). Cyclic AMP and cyclic GMP in boiled tissue extracts were determined by sensitive protein binding and radioimmunoassay techniques (see legend to Fig. 1).

Morphine (10<sup>-7</sup>–10<sup>-3</sup> M) increased the accumulation of cyclic GMP in rat striatal slices to a maximum of 200% of control values (Fig. 1). The tissue content of cyclic AMP was concomitantly decreased to about 80% of control, although this effect did not always attain statistical significance. Very similar control and morphine-stimulated values were obtained after isolation and separation of the two cyclic nucleotides by ion-exchange chromatography (Fig. 1 legend). The half-maximum response for both cyclic nucleotides occurred at a morphine concentration of about 3 × 10<sup>-8</sup> M. The dose–response curve for morphine was bell-shaped and higher morphine concentrations (10<sup>-4</sup>–10<sup>-3</sup> M) caused no significant changes in the tissue content of either cyclic nucleotide. Naloxone (10<sup>-6</sup> M) completely blocked the effects of morphine (10<sup>-6</sup> M) on the accumulation of both cyclic nucleotides (Table 1). Indeed, although naloxone alone had no action, in the presence of morphine and naloxone the accumulation of cyclic GMP was significantly reduced below control values (Table 1), although the tissue content of cyclic AMP was unaffected. Levorphanol, an

active congener of morphine at concentrations from 10<sup>-8</sup> to 10<sup>-3</sup> M mimicked the actions of morphine on cyclic nucleotide accumulation although the reduction in tissue cyclic AMP was not significant, whereas the inactive enantiomer dextrorphan tested at the same concentrations had no effect (Table 1). The latter observations suggest that the actions of morphine on cyclic nucleotide accumulation in rat striatal slices are due to an interaction with specific opiate receptors in this tissue.

The enkephalins are pentapeptides, isolated from porcine brain and characterised by Hughes *et al.*<sup>8</sup>, which seem to be specific opiate receptor agonists. Two similar



**Fig. 1** Effect of morphine on cyclic nucleotide concentrations in slices of rat neostriatum. The tissue preparation, incubation and extraction procedures were as described by Forn *et al.*<sup>7</sup>. After preincubation for 60 min at 37 °C, samples of neostriatal slices (260 × 260 μm; total 10 mg wet weight) were incubated for a further 15 min in the presence of various concentrations of morphine hydrochloride. Tissue extracts were prepared by boiling and centrifugation, 50 μl was taken for cyclic AMP determination by the method of Brown *et al.*<sup>11</sup>; and 100 μl was taken for determination of cyclic GMP by radioimmunoassay. The remaining tissue was dissolved in 1 ml 1 N NaOH and protein was measured by the method of Lowry *et al.*<sup>12</sup>. The cyclic GMP radioimmunoassay antiserum and method were provided by Dr J. Albano and will be described in detail elsewhere. The radioimmunoassay procedure was briefly as follows: 100 μl supernatant was added to 100 μl <sup>3</sup>H-cyclic GMP (0.02 μCi, 1.0 pmol) in 50 mM Tris-HCl (pH 7.4) containing 2 mM theophylline and 6 mM β-mercaptoethanol. Antiserum (100 μl) diluted in the same buffer was added and the tubes left at 2–4 °C for 6–18 h. The antibody complex was separated by membrane filtration; filters were washed with 5 ml of buffer and dissolved in 4 ml ethoxyethanol; 10 ml of 0.4% butyl PBD in toluene added and radioactivity determined. A standard curve for displacement of bound <sup>3</sup>H-cyclic GMP was determined using non-radioactive cyclic GMP. The sensitivity of the assay was 0.02 pmol cyclic GMP in 100 μl. Known amounts of both cyclic AMP and cyclic GMP added either separately or together as internal standards were quantitatively recovered, and the amount of each cyclic nucleotide measured was linearly related to the volume of supernatant used for assay. Cyclic GMP did not interfere with cyclic AMP determinations when present in up to a tenfold excess; cyclic AMP did not interfere with cyclic GMP when present in up to a 500-fold excess. In some experiments incubations were terminated by the addition of 1 ml 5% trichloroacetic acid, the tissue was homogenised and the cyclic nucleotides were isolated and separated by the method of Matsuzawa and Nirenberg<sup>13</sup>. All incubations were performed in quadruplicate, and the data are expressed as means ± s.e.m.

**Table 1** Effect of specific opiate narcotics and naloxone on cyclic nucleotide levels in slices of rat neostriatum

| Drug                                                         | Cyclic AMP<br>(pmol per<br>mg protein) | Cyclic GMP<br>(pmol per<br>mg protein) |
|--------------------------------------------------------------|----------------------------------------|----------------------------------------|
| Control                                                      | 9.1 ± 0.84                             | 0.15 ± 0.014                           |
| 10 <sup>-6</sup> M morphine                                  | 7.4 ± 0.26*                            | 0.28 ± 0.037†                          |
| 10 <sup>-6</sup> M naloxone                                  | 9.2 ± 0.31                             | 0.14 ± 0.016                           |
| 10 <sup>-6</sup> M morphine +<br>10 <sup>-6</sup> M naloxone | 9.1 ± 0.51                             | 0.10 ± 0.010*                          |
| 10 <sup>-6</sup> M levorphanol                               | 8.0 ± 0.71                             | 0.26 ± 0.036*                          |
| 10 <sup>-7</sup> M levorphanol                               | 7.6 ± 0.17                             | 0.29 ± 0.036†                          |
| 10 <sup>-6</sup> M dextrorphan                               | 9.2 ± 0.87                             | 0.16 ± 0.016                           |
| 10 <sup>-7</sup> M dextrorphan                               | 9.1 ± 0.28                             | 0.17 ± 0.019                           |

All values are means ± s.e.m. for four separate incubations, see Fig. 1 legend for details.

\**P* < 0.05.

†*P* < 0.01 compared with control values.

peptides exist, one with leucine as the C-terminal amino acid (leu-enkephalin) and one with methionine as the C-terminal amino acid (met-enkephalin). The addition of either of these compounds at concentrations up to 10<sup>-4</sup> M did not affect cyclic AMP or cyclic GMP accumulation in slices of rat neostriatum (Table 2). This result was, however, not unexpected, since both forms of enkephalin are rapidly inactivated in tissues at 37 °C (ref. 9). We also tested the effects of these substances in the presence of a peptidase inhibitor, bacitracin<sup>10</sup>, (30 µg ml<sup>-1</sup>) (Sigma Chemical Co.). At this concentration, bacitracin did not by itself significantly affect cyclic nucleotide levels nor did it interfere with the cyclic GMP response elicited by morphine (Table 2). In the presence of bacitracin, both leu- and met-enkephalin (10<sup>-4</sup> M) increased cyclic GMP accumulation in rat striatal slices to an extent similar to that seen with morphine (Table 2), and this effect was blocked by naloxone. Cyclic AMP accumulation was also decreased to a level similar to that observed in the presence of morphine (Table 2).

The effects of morphine on cyclic nucleotide accumulation in rat striatal slices are strikingly similar to those observed previously in neuroblastoma × glioma hybrid cells<sup>4</sup>, although the EC<sub>50</sub> for morphine is approximately one order of magnitude lower in striatal slices than in cell cultures, and the effect on both nucleotides is relatively greater in the cultured cells. Morphine displays a bell-shaped dose-response curve in both systems and in each case the

presence of morphine and naloxone significantly decreases cyclic GMP below control levels.

The potency of enkephalins (both leu- and met-) in displacing the specific binding of radiolabelled ligands from opiate receptors and on isolated test tissues is similar to that of morphine<sup>8</sup>. The fact that much higher concentrations of enkephalins are necessary to increase cyclic GMP levels in rat striatal slices probably reflects the instability of these substances in mammalian tissues and an incomplete inhibition of brain peptidases in our experiments. Higher concentrations of bacitracin were tested, but were found to interfere with the opiate response. It is, therefore, not yet possible to compare the true potencies of enkephalin and the opiate narcotics in this system. Nevertheless, the fact that the naturally occurring peptides, leu- and met-enkephalin increase cyclic GMP accumulation in rat striatal slices in the presence of a peptidase inhibitor raises the possibility that this response may also occur *in vivo*, and that it may represent a mechanism by which the actions of these substances, as well as those of morphine and other exogenously applied opiate narcotics are mediated at the cellular level.

We thank Dr J. Albano for the generous gift of cyclic GMP antiserum and Dr R. Miller for suggesting the use of bacitracin. Leu- and met-enkephalins were generously provided by Dr G. Metcalf, Reckitt and Colman Ltd, Hull, England; morphine, levorphanol and dextrorphan by Roche Products Ltd and naloxone by Endo Laboratories. K.P.M. is a graduate fellow of the US National Science Foundation.

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**Table 2** Effect of leu- and met-enkephalin on cyclic nucleotide levels in slices of rat neostriatum

| Drug(s)                                                                                                   | Cyclic AMP<br>(pmol per<br>mg protein) | Cyclic GMP<br>(pmol per<br>mg protein) |
|-----------------------------------------------------------------------------------------------------------|----------------------------------------|----------------------------------------|
| Control                                                                                                   | 8.1 ± 0.42                             | 0.14 ± 0.006                           |
| 10 <sup>-6</sup> M morphine                                                                               | 6.7 ± 0.91                             | 0.23 ± 0.014†                          |
| 10 <sup>-4</sup> M leu-enkephalin                                                                         | 8.1 ± 0.40                             | 0.15 ± 0.016                           |
| 10 <sup>-4</sup> M met-enkephalin                                                                         | 8.2 ± 0.43                             | 0.14 ± 0.011                           |
| 30 µg ml <sup>-1</sup> bacitracin                                                                         | 7.7 ± 0.29                             | 0.15 ± 0.015                           |
| 10 <sup>-6</sup> M morphine<br>+ 30 µg ml <sup>-1</sup> bacitracin                                        | 6.8 ± 0.32*                            | 0.22 ± 0.024*                          |
| 10 <sup>-4</sup> M leu-enkephalin<br>+ 30 µg ml <sup>-1</sup> bacitracin                                  | 6.7 ± 0.81                             | 0.22 ± 0.028*                          |
| 10 <sup>-4</sup> M met-enkephalin<br>+ 30 µg ml <sup>-1</sup> bacitracin                                  | 6.6 ± 0.12†                            | 0.25 ± 0.011†                          |
| 10 <sup>-4</sup> M leu-enkephalin<br>+ 30 µg ml <sup>-1</sup> bacitracin<br>+ 10 <sup>-6</sup> M naloxone | 8.0 ± 0.18                             | 0.14 ± 0.014                           |
| 10 <sup>-4</sup> M met-enkephalin<br>+ 30 µg ml <sup>-1</sup> bacitracin<br>+ 10 <sup>-6</sup> M naloxone | 7.5 ± 0.26                             | 0.13 ± 0.025                           |

All values are means ± s.e.m. for four separate incubations, see Fig. 1 legend for details.

\**P* < 0.05.

†*P* < 0.01 compared with control values.

## A sequential *in vivo-in vitro* study of carcinogenesis induced in the rat brain by ethylnitrosourea

THE processes which occur between exposure to a chemical carcinogen and the appearance of a gross tumour, the latent period, are still largely unknown. One approach to investigating how the biological properties of cells are altered during this time is to monitor the behaviour of cells cultured from target organs at different times after carcinogen treatment. A system in which a single dose of a chemical results in a high yield of tumours is a good starting point for such experiments. When pregnant rats are injected with ethylnitrosourea (ENU) almost every one of the progeny develops tumours of the nervous system<sup>1</sup>, of which macro- and microtumours of the brain constitute ~ 60% (predominantly cerebral gliomas<sup>2</sup>). To investigate the pro-



**Table 1** Agar growth and tumorigenicity of cultures derived 138–145 d after exposure to ENU or buffer

| Culture | Passage level | Average no. of colonies per 10 <sup>4</sup> cells plated | Plating efficiency in agar (%) | Passage level | No. of animals with tumours/No. injected | Average time before killed (d) |
|---------|---------------|----------------------------------------------------------|--------------------------------|---------------|------------------------------------------|--------------------------------|
| 22E     | 10            | 10                                                       | 0.1                            | 11            | 7/7                                      | 57.7                           |
| 22A     | 10            | 286                                                      | 2.9                            | 10            | 8/8                                      | 44.6                           |
| 23A     | 13            | 0                                                        | 0                              | 13            | 0/6                                      | > 265                          |
| 23C     | 10            | 0                                                        | 0                              |               |                                          | (1)                            |

To initiate cultures the brain tissue was sliced, chopped into small pieces (about 1 mm<sup>3</sup>) and incubated in 10 ml of dissociating medium (0.05% collagenase and 5% foetal calf serum in phosphate-buffered saline without divalent cations) at 37 °C for 20 min with occasional agitation. The suspension was dispersed with 10 or more excursions in a 10-ml pipette, centrifuged and resuspended in Dulbecco's modification of Eagle's medium with 15% foetal calf serum. The medium was changed on the following day to remove debris and unattached cells. Subculturing took place at weekly intervals soon after the cultures became confluent. To test for growth in agar, cells were plated at up to 10<sup>4</sup> in 1 ml of 0.3% Bacto-agar in Dulbecco's medium and 15% foetal calf serum. The base layer was 0.6% agar made up in the same medium. Two to four plates were prepared for each culture. Colonies of 22E and 22A were counted 2–4 weeks after plating, by which time many were macroscopic. The control cultures were observed for up to 3 months with feeding and were negative throughout. To test for tumorigenicity, cells were injected subcutaneously at  $1 \times 10^6$ – $2 \times 10^6$  per newborn animal (< 5 d old). Animals were examined three times each week and killed when the tumours were about 1 cm in diameter (1); 23C was injected a number of times but due to cannibalism only one animal survived from experiments at comparable passage levels. (It had no tumour at 104 d.) There was a large number of successful injections at later passages and these animals, together with those injected with 23A cells, are still under observation. Preliminary histological observation has indicated that the tumours formed by 22E and 22A are not fibrosarcomas, and have features of glial tumours. A more extensive examination is in progress and details will be published elsewhere.

cess of carcinogenesis during the latent period, cultures have been prepared from ENU-induced gliomas and from ENU-treated animals at different times before the appearance of such tumours.

Pregnant rats of the BD-IX inbred line were injected intraperitoneally with ENU at 40–50 mg per kg body weight on days 15 or 16 of pregnancy. The average latent period for brain tumours was 246 d. Cultures prepared from cerebral gliomas consisted mainly of cells resembling glia and cells with thin cytoplasm, indistinct boundaries and often what looked like incipient processes. We have called the latter type "basal layer cells"<sup>3,4</sup>. These are likely to be morphologically undifferentiated glia because they formed processes in response to dibutyladenosine cyclic monophosphate<sup>3</sup>, and because clones with a similar morphology have many of the characteristics of astrocytes<sup>4,5</sup>. The tumour cultures grew in 0.3% agar and were tumorigenic<sup>6</sup>.

For the initial set of experiments to investigate the latent period, cultures were prepared from a group of animals 138–145 d after transplacental exposure to either ENU or buffer. The cerebra of five ENU-treated and two buffer-treated animals were cultured. Two of the former cultures (22E, 22A) contained islands of cells resembling basal layer cells and associated glia characteristic of tumour cultures. There was no sign of a visible tumour during the slicing and chopping of brain tissue in either case. The two cultures were further investigated. The tumour-like cells which were initially in a minority, gradually became predominant on passaging. Both cultures contained cells which grew in agar and formed tumours in animals (Table 1). The cultures from buffer-exposed animals (23A, 23C) did not contain cells resembling cultured tumour cells, did not grow in agar and have not so far produced tumours (Table 1).

To investigate cells very early in the latent period, a culture was prepared from the pooled brains of an entire litter of foetuses 2 d after transplacental exposure to ENU

or buffer. The primary cultures from ENU-treated (BE10) and control (BE11) foetuses were morphologically indistinguishable. Tests for growth in agar and in animals were negative after many passages. At the 40th passage level, however, the BE10 culture formed small colonies, several weeks after plating in agar.

At later transfers macroscopic colonies developed in 2–4 weeks; the result of one experiment is given in Table 2. Further, two clones of BE10, BE10-13 and BE10-7 grew in agar and were tumorigenic (Table 2).

The culturing of cells shortly after treatment of an animal with a chemical as we have done in one set of experiments has been suggested as a test for carcinogens<sup>6</sup>. The method has also been used to ensure that target cells are affected by the ultimate carcinogen<sup>7,8</sup>, a situation not necessarily obtained by *in vitro* treatment of cultured cells. Kidney cells cultured 20 h to 7 d after the injection of rats with dimethylnitrosamine showed several transformed properties, but were not tumorigenic<sup>7</sup>. Laerum and Rajewsky<sup>8</sup> have reported that brain cultures made 20–90 h after transplacental exposure to ENU went through a series of changes. They describe the formation of piled-up foci of cells or 'nodules' which were washed off and cultured. These gave rise to cells which grew in 0.15% agar and became tumorigenic after a total of about 200 d in culture. Their control cultures died out before nodule formation so that no measure of spontaneous transformation could be made. The formation of foci which we have called 'clusters' has been observed in both ENU-treated and control cultures. These have been washed from the BE10 and BE11 cultures and maintained separately. There are some differences in the two cultures, for example, that from BE10 has a higher plating efficiency in agar (0.4–0.5%) than that from BE11 (0.01–0.1%) depending on passage level. Moreover, some of the animals injected several months before with the former have recently been found to have tumours. All four cultures

**Table 2** Agar growth and tumorigenicity of cultures derived 48 h after treatment with ENU or buffer

| Culture | Passage level | Average no. of colonies per 10 <sup>4</sup> cells plated | Plating efficiency in agar (%) | Passage level | No. of animals with tumours/No. injected | Average time before killed (d) |
|---------|---------------|----------------------------------------------------------|--------------------------------|---------------|------------------------------------------|--------------------------------|
| BE10    | 57            | 354/10 <sup>4</sup>                                      | 3.5                            | 60            |                                          | (1)                            |
| BE11    | 57            | <1/10 <sup>4</sup>                                       | <0.01                          | 60            |                                          | (1)                            |
| BE10-7  | 24            | 360/10 <sup>3</sup>                                      | 36                             | 28            | 8/8                                      | 95.1                           |
| BE10-13 | 32            | 54/10 <sup>4</sup>                                       | 0.5                            | 34            | 5/5                                      | 40                             |

BE10-13 was derived at the 10th and BE10-7 at the 20th transfer of BE10. This was before the earliest time at which cluster formation was observed in BE10. Other experimental details as in Table 1. (1) These animals are still under observation. The tumours formed by BE10-7 and BE10-13 are not fibrosarcomas and have glial features. Further histological observations are in progress and the results will be published elsewhere.



are being maintained and tested to estimate the extent of spontaneous transformation.

It seems, therefore, that at a time equivalent to about 3/5 of the average latent period, ENU-treated animals, have cells which, in culture, behave in a manner indistinguishable from tumour cells. These are not readily detected in cultures taken very early in the latent period. The results so far, however, suggest that there are cells present at 48 h with the potential to become tumorigenic, and that this potential is revealed after prolonged culturing. Experiments are in progress to try to detect these cells without such extensive culturing and to define the time at which committed cancer cells can first be readily detected in culture. It has recently been found that histological changes were first observed about 9 weeks after exposure of the foetuses to ENU (P. L. Lantos, personal communication). The combined *in vivo-in vitro* method used at a series of times in the latent period thus seems to be a valuable approach to investigating the process of carcinogenesis.

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## Native collagen formation by liver parenchymal cells in culture

In normal supporting tissue and repair tissue of animals collagen is usually considered to be produced by cells of mesenchymal origin. Little is known, however, about the type of cell which synthesises collagen in organs which undergo diffuse fibrosis without known physical trauma, such as in cirrhosis of the liver. Tissue culture has shown, however, that epithelial cells<sup>1</sup>, including cancer cells<sup>2</sup> and smooth muscle cells<sup>3</sup>, can synthesise collagen. We report here that an epithelial clone, originating from the liver cell line of rat forms a considerable amount of native collagen in culture.

An epithelial cell line from the liver of a JAR-2 suckling rat<sup>4</sup> was established as before<sup>5</sup>. The culture medium consisted of 0.5% lactalbumin hydrolysate medium (LE) supplemented with 10% calf serum (CS). After 200 d of culture the cells dispersed with 0.25% trypsin were seeded in 60-mm plastic Falcon plates for cloning. Inocula varied between a high of  $1 \times 10^3$  and a low of  $3 \times 10$  cells per plate, and the  $1 \times 10^3$  plate yielded colonies derived from single cells. Two colonies which were cytologically distinguishable from each other were picked out and cultured continuously. One consisted of rather small roundish cells and the other of larger polygonal cells. They were designated clones BB and BC, respectively. Both clones have been maintained by serial passages for 4 yr and 3 months in culture flasks containing Ham's F 12 medium and 10%

**Table 1** Production of serum proteins by cloned liver cell lines

| Culture generation | Al* (ng ml <sup>-1</sup> ) | AFP† (ng ml <sup>-1</sup> ) | Trans.‡ |
|--------------------|----------------------------|-----------------------------|---------|
| <b>BB cells</b>    |                            |                             |         |
| 8                  | 1,340                      | ND§                         | —       |
| 16                 | ND§                        | —                           | —       |
| 16                 | 270                        | —                           | —       |
| 25                 | ND§                        | ND§                         | (+)     |
| 25                 | 540                        | ND§                         | (-)     |
| 25                 | 570                        | 105                         | (-)     |
| 25                 | 670                        | ND§                         | (+)     |
| 41                 | ND§                        | ND§                         | —       |
| <b>BC cells</b>    |                            |                             |         |
| 9                  | 2,400                      | ND§                         | —       |
| 20                 | ND§                        | —                           | —       |
| 20                 | ND§                        | —                           | —       |
| 27                 | 340                        | ND§                         | (+)     |
| 27                 | 550                        | ND§                         | (-)     |
| 27                 | 420                        | ND§                         | (+)     |
| 27                 | 500                        | 82                          | (+)     |
| 43                 | ND§                        | ND§                         | —       |

The supernatants of culture media were collected 3-4 d after plating of cells and used for analysis with or without concentration.

\* Assays performed by Mancini's radial immunodiffusion technique<sup>6</sup> for 20-times concentrated samples. The sensitivity limit of this method is 300 ng ml<sup>-1</sup>.

† Values determined by radioimmunoassay<sup>7</sup> without concentration. The sensitivity limit is 20 ng ml<sup>-1</sup>.

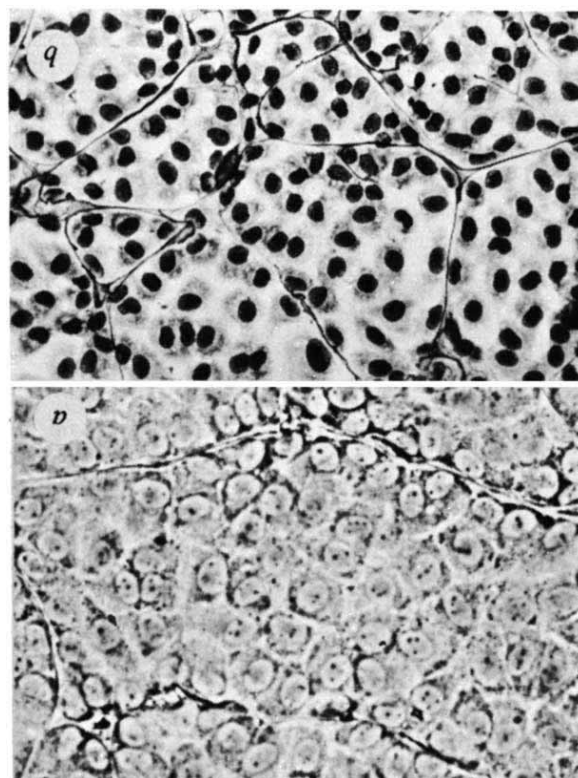
‡ Qualitative assays by immunautoradiography<sup>8</sup>.

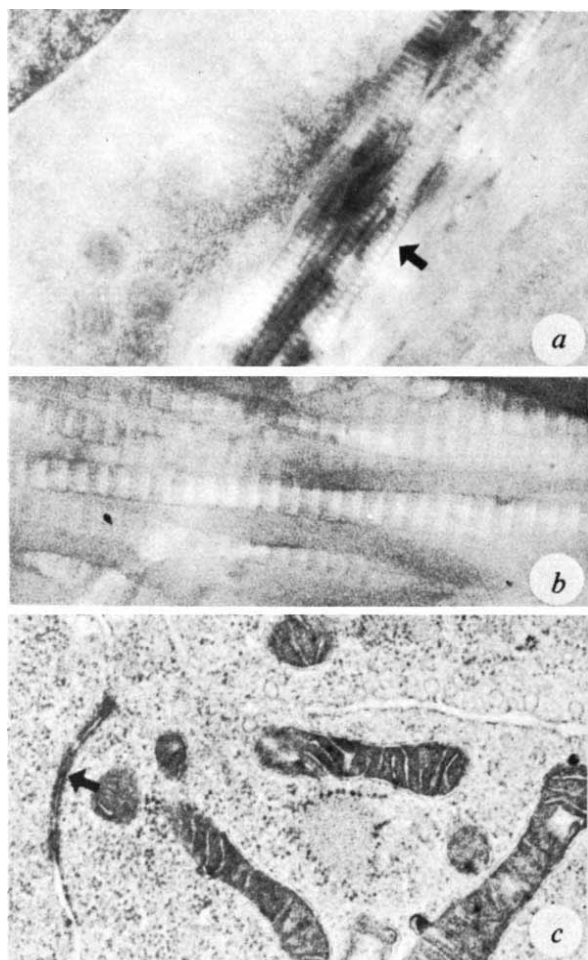
§ Non-detectable activity.

CS or foetal bovine serum (FBS). The cell replication cycle of these clones was 20-31 h after 21 generations. Plating efficiency was 35-55% after 19 generations. The cells were epithelioid, proliferated as monolayers, and achieved contact inhibition.

To detect liver-type metabolic activities of the cells, assays

**Fig. 1** Micrographs of a cloned liver cell line BC cultured on cover slip for 28 d without transfer. *a*, Unfixed, phase-contrast illumination ( $\times 587$ ); *b*, fixed with absolute methanol, stained by silver impregnation. Collagen fibres stained purple-brown situated between cells ( $\times 293$ ).





**Fig. 2** Electron micrographs of BC cells grown in the same conditions as described for Fig. 1. *a*, Bundle of collagen fibrils in the extracellular space (arrow), negatively stained with 20% phosphotungstic acid by the method of Kushida *et al.*<sup>10</sup> ( $\times 18,182$ ); *b*, higher magnification of collagen fibrils shown in *a*, demonstrating the banding pattern and periodicity of 640 Å ( $\times 45,455$ ); *c*, the portion of two adjacent cells illustrating the desmosome (arrow) and typical small in-pocketings of the plasma membrane, stained with uranyl acetate and lead citrate ( $\times 20,545$ ). Fixation, dehydration and embedding in Epon were as in the original plastic culture plates by the published procedure<sup>9</sup>.

of rat serum albumin (Al),  $\alpha$ -foetoprotein (AFP), and transferrin (trans.) in the culture fluid were performed after eight to 43 generations. Table 1 shows that these clones produced all three serum proteins, indicating that they were clones of rat liver parenchymal cells.

Although there was some difference in cytological appearances of the two clones, a particularly noticeable feature developed under the phase contrast microscope in BC cells left as a full sheet for more than 3 weeks without transfer. Thin fibre-like intercellular structures appeared, increased with time, began to enclose a portion of the culture, and finally divided the whole culture into multiple nodular areas (Fig. 1*a*). This process was usually complete within 6 weeks in our culture conditions.

To identify these structures we used silver impregnation, Mallory-Azan, resorcin-fuchsin, and periodic acid-Schiff. These staining methods, especially the former two, gave the characteristic colour reactions of collagen fibres (Fig. 1*b*). There was no collagen reaction in BB cells. Electron micrographs of BC cells revealed fibrils of characteristic collagen periodicity, deposited in broad intercellular spaces which

separated the cells from each other (Fig. 2*a* and *b*). Where cell perimeters were in contact they were connected tightly to desmosomes, arranged in a direction in which collagen fibrils were rarely found (Fig. 2*c*).

To preclude the possibility of contamination of fibroblasts in the original BC clone, we recloned it. Three subclones, BC.S1, BC.S2, and BC.S3, were obtained from plates seeded with  $1 \times 10^2$  cells by the same procedure as before. The subclones were morphologically similar to the parent clone. For quantitative proof of these findings a hydroxyproline assay of the cell layers maintained for 21 d without subculture was performed by the method of Prockop and Udenfriend<sup>11</sup>. Table 2 shows that values obtained from clone BC and its subclones were more than five times greater than that from the mouse fibroblast line 3T3 (Table 2), for which electron microscopy had shown evidence of production of collagen in extremely small amounts<sup>9</sup>.

To see whether the cells had undergone spontaneous malignant transformation, we tested colony-forming capacity in soft agar, tumorigenicity in the cheek pouches of golden hamsters treated with antithymocyte serum, and chromosome number between 48 and 53 generations. No

**Table 2** Hydroxyproline in cell layers after 21 d in culture without transfer

| Cells    | nmol per $10^7$ cells |
|----------|-----------------------|
| BC       | 152                   |
| BC.S1    | 123                   |
| BC.S2    | 139                   |
| BC.S3    | 98                    |
| Embryo*  | 184                   |
| 3T3†     | 18                    |
| HeLa.S3‡ | <10                   |

Cells growing in monolayer were scraped from the plates, washed twice with Hanks' solution by centrifugation at 1,500 r.p.m. for 5 min, hydrolysed at 110 °C for 24 h, and hydroxyproline was determined. For each determination trypsinised replicate cultures were used for cell counts.

\* Primary culture of the whole embryo of JAR-2 strain rat as a positive control of collagen synthesis *in vitro*.

† A mouse fibroblast line<sup>12</sup>.

‡ A cloned subline of HeLa, human uterine cancer cell line<sup>13</sup>. Assay for this cell line was exceptionally performed after 10 d in culture.

colony developed in soft agar, nor did the cheek pouches of the hamsters reveal any sign of tumours. The predominant mode of chromosome number was 59, indicating hypotriploidy.

Our data demonstrate the production of native collagen by one of two clonal lines of rat liver cells. Both had synthesised *in vitro* three proteins which are regarded as the differential products of liver parenchyma in early culture generations.

The question arises whether collagen synthesis is a normal function of liver parenchymal cells. It is widely believed that collagen fibres in the liver are formed by the sinusoid lining cells and the fibroblasts of Glisson's capsule.

There are, however, data suggesting that liver parenchymal cells participate in collagen synthesis *in vivo*<sup>14</sup>: 100 times more procollagen proline hydroxylase activity was reported in the parenchymal cell fraction than in the mesenchymal cell fraction, both of which were isolated from the same adult rat liver. Our results support these findings. They also enable us to speculate that liver parenchymal cells produce the collagen fibres found in the space of Disse, overproduction of which is normally controlled by a homeostatic mechanism. We suggest that the marked increase of collagen fibres in liver cirrhosis represents a failure of this mechanism.

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## Identification of medicarpin as a phytoalexin in the broad bean plant (*Vicia faba* L.)

THE analysis of phytoalexin induction has been proposed as a dynamic approach to plant systematics<sup>1</sup>. It has also been pointed out that production of the furanoacetylene wyerone acid<sup>2</sup> by *Vicia faba* is an intriguing anomaly to the more general accumulation of isoflavanoid phytoalexins by members of the Leguminosae. In our studies on phytoalexin production by *V. faba*, three related furanoacetylenes (Fig. 1), wyerone acid (a), wyerone (b)<sup>3</sup> and wyerone epoxide (c)<sup>4</sup>, have been identified as the major components of the multiple phytoalexin response of the broad bean plant to fungal infection<sup>5</sup>. In addition to these phytoalexins other minor inhibitors were detected in bioassays of thin layer chromatograms of methanol extracts of infected tissues<sup>6</sup>. Only one of these inhibitors reacted with *p*-nitroaniline<sup>6</sup> to give an orange coloration indicating phenolic character. No anti-fungal compounds were detected in extracts of healthy tissues. Here we report the identification of the phenolic inhibitor as medicarpin (3-hydroxy-9-methoxy pterocarpin) (Fig. 1d), a phytoalexin present in a wide range of leguminous species<sup>1</sup>.

Fig. 1 a, Wyerone acid (R = H); b, wyerone (R = Me); c, wyerone epoxide; d, medicarpin

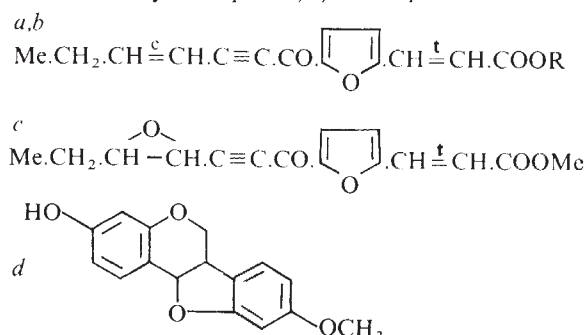


Table 1 Yields of phytoalexins from *V. faba* pod endocarp tissue bearing limited lesions caused by *B. cinerea* 4 d after fungal inoculation

| Phytoalexin     | Yield (μg per g fresh tissue) |
|-----------------|-------------------------------|
| Wyerone         | 217                           |
| Wyerone acid    | 219                           |
| Wyerone epoxide | 30.7                          |
| Medicarpin      | 19.5                          |

Yields are the mean of results from two experiments. Samples (about 4 g) of infected tissue were macerated in diethyl ether and the ether extract subjected to thin-layer chromatography (TLC) on precoated plates (Merck 5715). Wyerone acid was isolated after TLC in ether-methanol (6:1) and like other wyerone derivatives was recognised by its blue fluorescence under ultraviolet light (366 nm) at  $R_F$  0.53. TLC of extracts in hexane-acetone (2:1) followed by chloroform-petrol (2:1) enabled purification of wyerone ( $R_F$  0.6), wyerone epoxide ( $R_F$  0.53) and medicarpin ( $R_F$  0.31); the latter quenched the fluorescence of silica gel under ultraviolet light (254 nm). After elution in methanol concentrations of the phytoalexins were recorded by ultraviolet spectrophotometry<sup>2,4</sup>.

Pod endocarp tissue (2.5 kg) bearing limited lesions caused by *Botrytis cinerea* was collected 5 d after fungal inoculation<sup>7</sup>. Diethyl ether extracts of the infected tissue<sup>3</sup> were subjected to preparative layer chromatography (PLC) on silica gel (Merck GF254 type 60) using hexane-acetone (2:1) followed after drying with chloroform-petroleum spirit 60–80 °C (2:1) as solvents. The band giving a positive *p*-nitroaniline test ( $R_F$  0.34) was eluted and the phenolic compound repurified by PLC in chloroform ( $R_F$  0.35) and finally chloroform-methanol (10:1).

After elution from  $R_F$  0.6 the inhibitor (7 mg) was crystallised from ether-hexane. Proton magnetic resonance spectra of the crystalline product in deuterochloroform were consistent with structure d in Fig. 1. Recorded optical activity ( $[\alpha]_D^{20}$  –214°  $c$  = 0.19, chloroform, melting point 129–130 °C); and ultraviolet absorption spectrum  $\lambda_{\text{max}}$  (ethanol) 287 (log  $\epsilon$  = 3.94), 282 (3.88), 226 (4.14), 210 nm (4.53) were virtually identical to those published for medicarpin<sup>8</sup>. Identification was confirmed by comparison of the mass spectra obtained for our sample and authentic medicarpin.

To date *Vicia faba* seems to be unique in its *de novo* production of chemically unrelated compounds which act as phytoalexins. Much lower concentrations of medicarpin than of wyerone derivatives accumulated within infected tissue (Table 1). This suggests that medicarpin is probably not of great significance to the inhibition of growth of invading fungal hyphae. Medicarpin biosynthesis in *V. faba* may represent the expression of a primitive resistance response which has been largely superseded by the accumulation of the furanoacetylenes. Preliminary examination of the production of wyerone derivatives in pod tissues of different species of *Vicia* in response to infection by *B. cinerea* indicated that the more primitive<sup>9</sup> *V. cracca*, *V. sativa*, *V. sepium* and *V. sylvatica* do not produce wyerone or wyerone acid. These inhibitors, however, were induced in pods of *V. galilea* and *V. narbonensis*, two species closely related to *V. faba*.

Our results provide further support for the proposal that analysis of phytoalexin induction may become an invaluable approach to plant systematic and phylogenetic relationships. It should prove worthwhile to examine medicarpin and furanoacetylene induction within the genus *Vicia*. Note, however, that in our experience, investigation of phytoalexin accumulation within infection droplets<sup>1</sup> does not provide a complete picture of phytoalexin induction. It is only by thorough analysis of infected tissue that the more subtle relationships are likely to be discerned. For example, we have detected only trace amounts of medicarpin and wyerone within infection droplets containing conidia of *B. cinerea* incubated on leaves or pod seed cavities of *V. faba*.

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## Initiation inhibition and reinitiation of the synthesis of heterogeneous nuclear RNA in living cells

THE nucleoside analogue 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) inhibits the synthesis of heterogeneous nuclear RNA (hnRNA) at the initiation level in explanted salivary glands of *Chironomus tentans*, but does not interfere with the synthesis of ribosomal RNA and transfer RNA (refs 1–4). When the radioactive RNA precursors and the inhibitor are coincidentally added to the glands the labelling of hnRNA transcripts produced by smaller transcriptional units is suppressed before that of the larger ones<sup>2,3</sup>. If, however, the glands are pretreated with DRB, and the time of preincubation is sufficiently long, labelling of small as well as large hnRNA transcripts is equally eliminated, and the DNA template becomes depleted of nascent hnRNA chains<sup>2–4</sup>. Important questions, arising from these findings, are whether DRB can be washed out from the cells, and whether a reinitiation of hnRNA synthesis can subsequently be achieved. This paper presents data which show that the initiation of the RNA synthesis resumes when glands pretreated with DRB are washed and replenished with inhibitor-free medium.

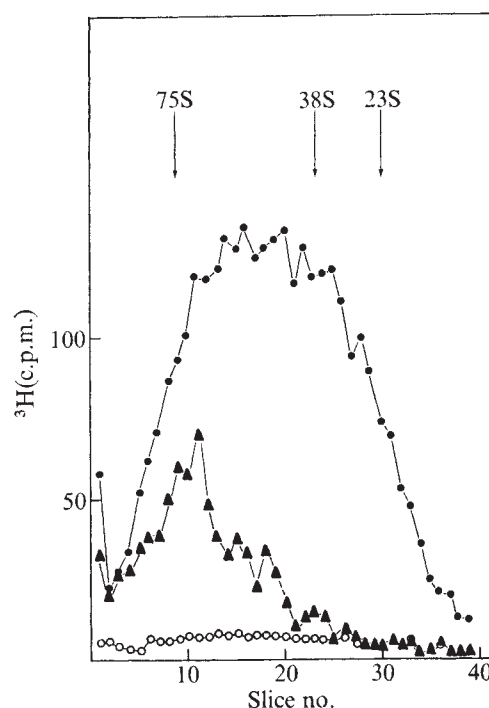
A reversal of transcription inhibition by DRB should result in a preferential labelling of hnRNA chains in the lower molecular weight ranges, if the incubation period after removal of DRB is brief enough (< 15–30 min). Labelled molecules in the 75–100S range could be expected only around 30 min (calculated synthesis time of the largest hnRNA molecules) after transferring inhibited glands to a DRB-free medium. A reversal of the effect of DRB on polyinosinicpolycytidylic acid-induced interferon production in a strain of diploid human fibroblast has been reported<sup>5</sup>. The superinducing action of DRB was promptly abolished by removing DRB from the cells. The reversibility of the transcription by DRB also increases its usefulness as a tool in studies of transcription mechanism and metabolism of hnRNA in living cells.

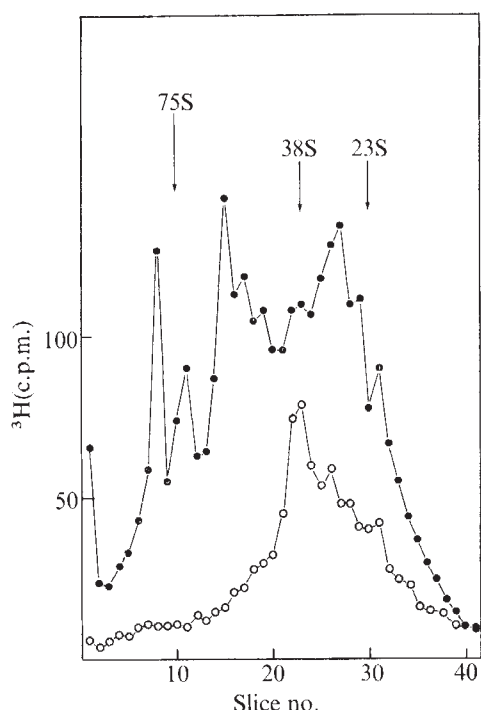
In an attempt to examine whether DRB is a reversible inhibitor of the synthesis of chromosomal hnRNA and to record the sequence of hnRNA events after a possible reinitiation of the transcription, explanted glands were preincubated with DRB in 50  $\mu$ l of Cannon's modified insect medium for 30 min at 18 °C (refs 6 and 7). The glands were then washed and replenished with inhibitor-free medium supplied with <sup>3</sup>H-cytidine and <sup>3</sup>H-uridine and were incubated

for different periods. Fixation of glands, microdissection of chromosomes, extraction and electrophoresis of RNA and the measurement of radioactivity were carried out as described elsewhere<sup>2–4,8</sup>. The RNA of chromosome IV, dominated by Balbiani ring 1 and 2 RNA, shows labelling kinetics different from those of chromosomes I to III (refs 2–4), and therefore the present analyses of chromosomal RNA do not include RNA from chromosome IV.

To demonstrate the effect of DRB on the initiation of hnRNA synthesis in chromosomes I to III, explanted glands were preincubated with DRB for 15 and 30 min before labelling for 20 min with tritiated cytidine and uridine in the continued presence of the analogue (Fig. 1). The control glands were labelled in similar conditions, but without inhibitor. The electrophoretic profile of labelled chromosomal hnRNA from control cells shows a heterogeneous distribution of molecules in the 16–100S range as described earlier<sup>2,3,9</sup>. When DRB was added to the incubation system 15 min before isotopic nucleosides, there was an almost complete disappearance of label in the 23–38S range of hnRNA, but incorporation into the 75–100S region was considerably less affected, as observed previously. If, however, the preincubation period with DRB, before administration of the tritiated RNA precursors, was prolonged from 15 to 30 min, the synthesis of short hnRNA molecules as well as of

**Fig. 1** Electrophoretic analysis of chromosomal hnRNA labelled for 20 min in the absence and in the presence of DRB after preincubation in the presence of DRB. Salivary glands were incubated at 18 °C for 15 and 30 min in 50  $\mu$ l of incubation medium containing DRB (65  $\mu$ M). They were then transferred to another 50  $\mu$ l of the same medium containing DRB, 100  $\mu$ Ci of <sup>3</sup>H-cytidine at 29 Ci mmol<sup>-1</sup> and 200  $\mu$ Ci of <sup>3</sup>H-uridine at 44 Ci mmol<sup>-1</sup> (Amersham) and incubated for 20 min. Labelling with tritiated nucleosides in the absence of DRB was carried out in parallel. For labelling with isotopic nucleosides after preincubation for 15 min (● and ▲) sister glands were used. Chromosomes I to III were microdissected from 20 cells (four glands). The labelled RNA from each sample was released by Pronase-sodium dodecyl sulphate treatment, and the electrophoresis was carried out in 1% Agarose gel. Before radioactivity measurement, the gel was sliced and the slices counted in a Packard liquid scintillation spectrometer (Model 3380). *E. coli* RNA was used as marker. The position of 75S and 38S were determined in parallel analyses of BR 2 RNA and nucleolar RNA, respectively. ●, Normal cells; ▲, 15 min of preincubation in DRB; ○, 30 min of preincubation in DRB.





**Fig. 2** Electrophoretic analyses of chromosomal hnRNA labelled for 15 min without preincubation in DRB, and after preincubation in DRB followed by removal of DRB from the cells before addition of isotopic nucleosides. Four glands were incubated at 18 °C for 30 min in 50  $\mu$ l of incubation medium containing DRB (65  $\mu$ M). The glands were then transferred to an inhibitor-free medium, and were rinsed 3 times each in a volume of 0.5 ml. They were then placed in 50  $\mu$ l of DRB-free medium containing 100  $\mu$ Ci of  $^3$ H-cytidine and 200  $\mu$ Ci of  $^3$ H-uridine and incubated for 15 min. For labelling with tritiated nucleosides without preincubation with DRB (control glands), the sister glands were used in an otherwise parallel procedure. Chromosomes I to III were dissected from 20 cells (four glands). For other data see the legend to Fig. 1. ●, Normal cells; ○, DRB-treated cells.

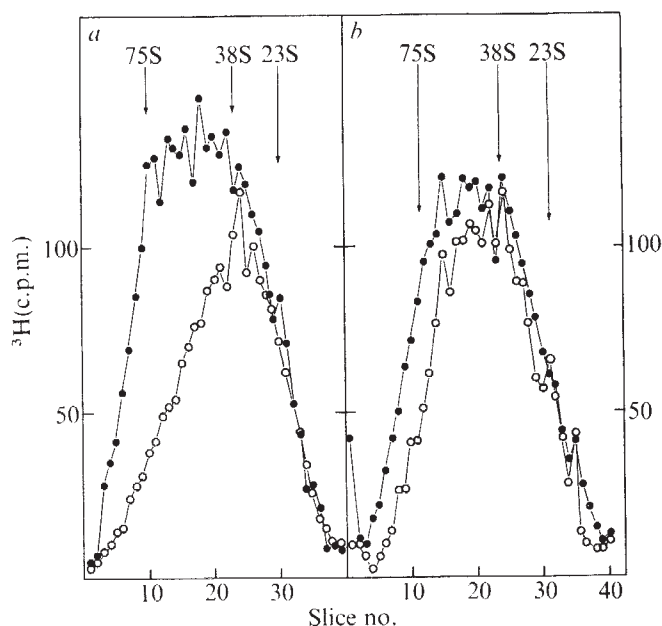
long molecules was equally interrupted, though 5–10% of the labelled material, distributed evenly over the whole spectrum, survived the drug treatment. On the basis of these and previous observations<sup>2,4</sup>, it seems likely that the DNA segments which serve as template for hnRNA synthesis are essentially cleared of nascent RNA chains 30 min after addition of DRB.

An efficient reinitiation of transcription after inhibition by DRB is conditional on first, that the intracellular DRB concentration is rapidly reducible to a level not being inhibitory; and second, that the interference of DRB or DRB metabolite (presumably a DRB triphosphate) with the initiation mechanism is reversible after removal of the compound from the cells. Experiments were designed to clear the DNA template of nascent hnRNA chains by blocking the initiation of new chains with DRB for 30 min, and then to reverse the inhibition of initiation simply by washing out DRB from the cells. After rinsing in excess volumes of incubation medium lacking the inhibitor (3  $\times$  0.5 ml), the glands were incubated for 15 min in a drug-free medium supplemented with tritiated cytidine and uridine (Fig. 2). The control glands were incubated for the same period but without DRB throughout. Although the electrophoretic analysis of hnRNA derived from control cells shows heterogeneous distribution of label in the 16–100S range<sup>2,3</sup>, the corresponding analysis of hnRNA from DRB-pretreated and washed glands shows essentially one broad and somewhat asymmetric peak in the 23–38S region. It has a rather steep slope on the high molecular weight side, but is less steep on the other side. Thus, 15 min after removal of DRB from pretreated glands, transcription inhibition seems to be reversed, and a reinitiation of hnRNA synthesis has been taking place. The maximum sizes of hnRNA chains initiated and elongated after removal of inhibitor are, however, signifi-

cantly less than that of hnRNA produced by cells with an uninterrupted and continuous initiation of transcription. Most of the labelled chains have a molecular size not exceeding that of the 38S marker, although there is also some label in the 38–100S range. This latter material can probably be attributed to radioactivity associated with growing chains which were not eliminated during pretreatment with DRB (refs 1, 2 and Fig. 1). Such uninhibited RNA chains of the larger transcriptional units are then able to produce labelled hnRNA molecules in the 38–100S range immediately after administration of labelled RNA precursors irrespective of whether or not reinitiation is taking place.

To follow the sequence of hnRNA events after drug treatment and replenishment with drug-free medium, isotopic nucleosides were added to the glands 5 min (Fig. 3a) and 30 min (Fig. 3b) after removal of DRB, and the incorporation continued for 20 min. Control glands were incubated in a similar way but in the absence of inhibitor throughout incubation. The electrophoretic profile of chromosomal hnRNA, derived from glands to which tritiated nucleosides were introduced 5 min after removal of DRB, agrees reasonably well with that of hnRNA extracted from control cells in the range below the 38S marker (Fig. 3a). The amount of labelled material in the 38–100S range, however, is lower in treated cells, decreasing gradually from the 38S region towards the 100S range. In the 75–100S region, it comprises 30–40% of the radioactivity level found in the normal pattern. Considering that escape labelling of hnRNA during DRB treatment constitutes not more than 5–10% of the uninterrupted RNA labelling, it seems probable that reinitiated 75–100S RNA molecules have attained their maximum size (finished transcripts) 25 min after reversal of inhibitory action. It should be observed, however, that even though reinitiated hnRNA chains in the 75–100S range have been completed 25 min after removal of DRB, the amount of labelled

**Fig. 3** Electrophoretic analyses of chromosomal hnRNA labelled for 20 min without preincubation in DRB, and after preincubation in DRB followed by removal of DRB from the cells before addition of isotopic nucleosides. In each experiment four glands from four different animals were incubated at 18 °C for 30 min in 50  $\mu$ l of incubation medium containing DRB (65  $\mu$ M). The glands were then transferred to an inhibitor-free medium and rinsed 3 times each in a volume of 0.5 ml and placed in 50  $\mu$ l of fresh medium for a, 5 and b, 30 min. Next, 100  $\mu$ Ci of  $^3$ H-cytidine and 200  $\mu$ Ci of  $^3$ H-uridine were introduced to the medium and the glands were labelled for 20 min. For labelling with tritiated nucleosides without preincubation in DRB (control glands), the sister glands were used in an otherwise parallel procedure. Chromosomes I to III were dissected from 20 cells (four glands). For other details see the legend to Fig. 1. ●, Normal cells; ○, DRB-treated cells.





RNA in this part of the spectrum is, still 60–70% lower than that in untreated cells. This may be because: (1) transcription initiation is not restored to normal efficiency after replacement of medium containing DRB with inhibitor-free medium (partial reinitiation); and/or (2) finished hnRNA molecules accumulate after their completion on chromosomes I to III. The latter would imply that chromosomal RNA extracts from control cells contain a larger quantity of finished molecules than that from cells which were pretreated with DRB for 30 min and incubated in inhibitor-free medium for 25 min after removal of DRB (Fig. 3a).

The finding that hnRNA molecules migrating faster than 38S display a normal rate of labelling if isotopic nucleosides are added 5 min after transferring glands to drug-free medium (Fig. 3a) argues against inefficiency in transcription reinitiation. Previous observations that hnRNA on the chromosomes I to III attains maximum specific activity after 45–90 min of incorporation<sup>2</sup>, whereas the labelling of Balbiani ring RNA of similar size reaches a plateau value after 20 min of labelling<sup>10</sup> are compatible with an accumulation of hnRNA molecules on the chromosomes I to III after their completion. Therefore, to restore the rate of labelling and accumulation of chromosomal 75–100S RNA to the normal condition after removal of DRB from inhibited cells, incubation for 20 min or longer would be required before the addition of radioactive RNA precursors. In the experiment shown in Fig. 3b, the interval between washing and replenishing DRB-treated glands with drug-free medium and administration of isotopic nucleosides was prolonged to about 30 min. As predicted, the electrophoretic patterns of chromosomal hnRNA derived from inhibited and washed cells and from untreated cells display a reasonably good agreement, although absolute equality cannot be claimed. The results in Fig. 3 strongly indicate that 25 min of incubation after washing out DRB from cells (5 min in the absence plus 20 min in the presence of radioactive nucleosides) pretreated with the drug for 30 min, is sufficiently long to reinitiate, elongate and complete hnRNA chains migrating as 75–100S RNA. To bring the rate of the synthesis (accumulation) of the largest hnRNA molecules on the chromosomes I to III to a level similar to that before exposure to transcription inhibition, however, incubation in inhibitor-free medium for at least 30 min is demanded after removal of DRB. The principal reason for that is probably the accumulation of newly made hnRNA on chromosomes I to III rather than, or in addition to, an inefficient reinitiation of the transcription.

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## Inactivation of parathyroid hormone mRNA by treatment with periodate and aniline

TRANSLATION of parathyroid hormone (PTH) mRNA in extracts of wheat germ<sup>1</sup> or Krebs II ascites cells<sup>2</sup> produces a polypeptide which contains the structure of PTH but which is larger than either PTH or its biosynthetic precursor preproparathyroid hormone<sup>3,4</sup> (proPTH). In the wheat germ

system, this cell-free protein, designated pre-proparathyroid hormone (pre-proPTH) contains a methionine at the amino terminus<sup>5</sup> derived from initiator methionyl-tRNA<sup>6</sup>. Although this observation suggests that initiation of the synthesis of pre-proPTH is normal in the cell-free system, it is possible that *in vitro* initiation occurs at a codon that codes for an internal methionine *in vivo*. Such an artefact could be due to a degraded mRNA in which the *in vivo* initiator codon is lost or inactivated.

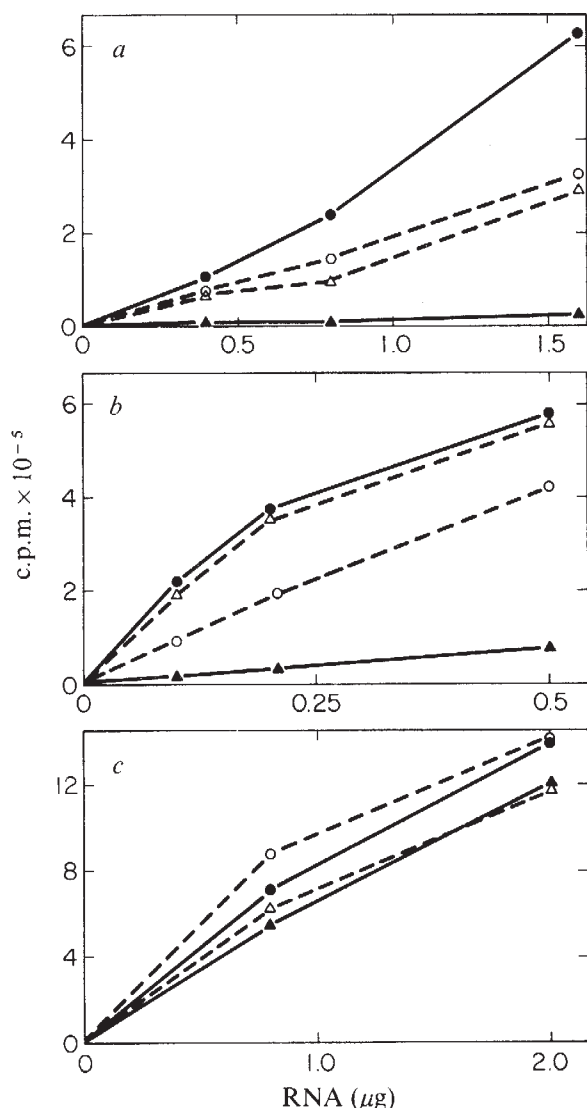
Recent studies on the structure of mRNA have suggested a method for demonstrating that an isolated mRNA is intact at the 5' end and thus that the proper initiator codon is present. Many mRNAs of viruses and eukaryotic cells have been shown to be blocked at the 5' end by a 7-methylguanosine linked through its 5' hydroxyl by way of a triphosphate or pyrophosphate to the 5' hydroxyl of the penultimate nucleoside<sup>7</sup>. This 5'–5' linked structure is necessary for the translation of mRNAs of vesicular stomatitis virus, reovirus, and haemoglobin<sup>8,9</sup> and mRNA from brine shrimp<sup>10</sup>. The 7-methylguanosine can be removed from the mRNAs by oxidation with periodate followed by a  $\beta$  elimination reaction catalysed by aniline<sup>9</sup>. I have compared the effects of periodate and aniline treatment on the activity of PTH mRNA with the effects on the activities of haemoglobin mRNA which has a blocked 5' terminus and with satellite tobacco necrosis virus (STNV) mRNA which does not contain a methylated blocked 5' terminus<sup>11,12</sup> and is actively translated in the wheat germ cell-free protein synthesising system without being methylated (J. M. Clark, Jr, personal communication).

Treatment with periodate followed by aniline inhibited the translation of PTH and haemoglobin mRNAs by 90% or more, compared with controls incubated in the same manner without periodate and aniline (Fig. 1a and b). In contrast, the activity of STNV mRNA was inhibited only about 15% by this treatment, an inhibition similar to that observed in the control incubated with aniline alone (Fig. 1c). Treatment with periodate alone substantially reduced the activity of haemoglobin and PTH mRNA but had no inhibitory effect on STNV RNA. The resistance of STNV RNA to this treatment minimises the possibility that the periodate inhibition is a nonspecific effect. The inhibition may be due to spontaneous  $\beta$  elimination of the oxidised mRNA, or the oxidation of the sugar moiety on methylguanosine may alter the 5' end enough to decrease the efficiency of initiation. The present data do not allow us to distinguish between these two possibilities. Rao *et al.*<sup>13</sup>, however, have shown that periodate treatment alone completely inhibits the activity of rat liver mRNA and have concluded that the unoxidised ribose is required for activity. It is not clear why they observed nearly 100% inhibition after periodate oxidation and I observed only a 50% inhibition.

The effect of aniline treatment alone on the activity of the mRNAs was somewhat variable. The activity of haemoglobin mRNA was not reduced by aniline treatment while PTH mRNA and STNV RNA were inhibited by about 50 and 15%, respectively. Steinschneider and Frankel-Conrat have reported that aniline treatment alone reduced the infectivity of tobacco mosaic virus RNA by 50% and that some aniline was tightly bound to the RNA and could not be removed by extensive washing<sup>14</sup>. Thus, different mRNA may be differentially sensitive to treatment by aniline.

Although treatment with either periodate or aniline alone reduced the activity of PTH mRNA, the inhibition after treatment with both chemicals was more than simply a summation of the two individual inhibitions. In the experiment shown in Fig. 1a, the sample treated with aniline and periodate should have been about 25% of control if the effects of aniline and periodate were additive instead of the observed 4% of control. In another experiment the





**Fig. 1** Translation in wheat germ extracts of PTH (a), haemoglobin (b) and STNV (c) mRNAs after treatment with periodate and aniline. Haemoglobin mRNA was isolated as described by Labrie<sup>15</sup>. Parathyroid RNA sedimenting between 8S and 14S (designated PTH mRNA in this report) was isolated as described previously<sup>1</sup> with modifications. Bovine parathyroid glands were obtained at the time of slaughter and were stored in liquid nitrogen. About 1 g of glands was sliced and then homogenised for 1 min in a Waring blender in 5 ml of phenol, 5 ml of chloroform containing 4% isoamyl alcohol and 2 ml of 10% sodium dodecyl sulphate. 5 ml of buffer (0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.001 M EDTA, 0.1% sodium dodecyl sulphate) was added and RNA was extracted by shaking with phenol-chloroform as described previously<sup>1</sup>. DNA and low molecular weight RNA were extracted from the sample with 3 M sodium acetate, pH 6.0, 7.5 mM EDTA<sup>16</sup> and the RNA sedimenting on a sucrose gradient from 8S to 14S was isolated from the high molecular weight RNA as before<sup>1</sup>. The mRNAs were incubated with periodate and aniline as described by Steinschneider and Fraenkel-Conrat<sup>14</sup> and modified by Mayer *et al.*<sup>17</sup>. 10  $\mu$ g of haemoglobin or STNV mRNA or 4  $\mu$ g of PTH mRNA were incubated in 50  $\mu$ l of 0.1 M sodium acetate, pH 5.3, 1 mM EDTA containing 1 mM sodium periodate for 2 h at room temperature in the dark. Glucose was added to 10 mM, 150  $\mu$ l ethanol were added and RNA was precipitated overnight at  $-20^{\circ}\text{C}$ . The RNA was centrifuged in a Brinkman microfuge, washed with ethanol, dried under vacuum and dissolved in 50  $\mu$ l of 0.33 M aniline HCl. After incubation for 2 h at room temperature in the dark, NaCl was added to 0.3 M and 150  $\mu$ l of ethanol were added. The samples were left overnight at  $-20^{\circ}\text{C}$ , and the RNA was centrifuged, washed twice with ethanol, dried under vacuum and dissolved in 25  $\mu$ l of  $\text{H}_2\text{O}$  for translation in the wheat germ extracts. Controls were incubated in the same manner except that either aniline or periodate or both were not added. Conditions for the translation of the mRNA in the wheat germ cell-free system have been described<sup>1</sup>. 5–10  $\mu\text{Ci}$  of  $^{35}\text{S}$ -methionine (300 Ci mmol<sup>-1</sup>, Amersham Searle) were added to each reaction of 25  $\mu$ l. Total trichloroacetic acid-insoluble radioactivity was determined in 1- or 2-  $\mu$ l aliquots of the reaction. The amounts of radioactivity and RNA indicated in the figure are the c.p.m. and  $\mu\text{g}$ , respectively, in a reaction of 25  $\mu$ l. The amount of radioactivity (a,  $0.43 \times 10^5$  c.p.m.; b,  $0.26 \times 10^5$  c.p.m.; and c,  $0.45 \times 10^5$ ) incorporated into protein if mRNA was not added to the reaction was subtracted from each of the plotted values. ●, Incubated without aniline or periodate; △, incubated with aniline and without periodate; ○, incubated with periodate and without aniline; ▲, incubated with periodate and aniline.

treatment with aniline and periodate was reversed. The samples treated with either periodate or aniline alone were 73% and 85% of control, respectively. The sample treated in reverse order with aniline and periodate was 54% of control, near the 62% expected as a result of simple additive effects, while the sample treated with periodate and then aniline was 19% of control. The inhibition was less in this experiment than that shown in Fig. 1, probably because 0.27 M aniline was used instead of 0.33 M aniline. This synergistic action suggests that the effect of the periodate/aniline treatment is the result of a  $\beta$ -elimination reaction which would be expected if PTH mRNA has a nucleoside in 5'-5' linkage at the 5' end.

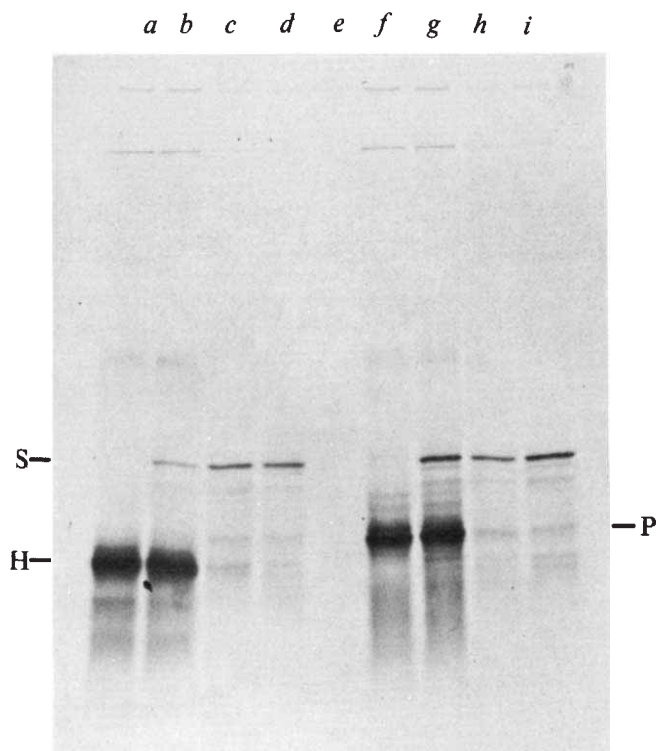
Analysis of the products of the translation of the mRNAs in the wheat germ extracts by sodium dodecyl sulphate slab gel electrophoresis is shown in Fig. 2. Each of the mRNAs directs primarily the synthesis of the expected protein products. If STNV RNA is mixed with either PTH mRNA or haemoglobin mRNA, both the RNAs in the mixture direct the synthesis of their respective proteins (Fig. 2b and g) but after treatment of the mixture with periodate and aniline, essentially only STNV proteins are observed (Fig. 2c and h).

These experiments demonstrate that inactivation of mRNAs by treatment with periodate and aniline is an effective means of determining whether mRNAs contain a 5'-5' linked nucleoside at the 5' terminus, particularly with

an impure preparation of mRNA such as that used in this report for PTH mRNA when direct chemical analysis is not possible. The inactivation of haemoglobin and PTH mRNAs by this treatment supports studies with other mRNAs indicating that the blocked 5' end is required for maximal activity of mRNA<sup>8-10</sup>. Simply demonstrating that periodate-aniline treatment causes inhibition of mRNA activity is not sufficient to conclude that a particular mRNA contains such a 5'-5' linked nucleoside since treatment with aniline or periodate alone can cause substantial inhibition. The controls of treatment with aniline or periodate alone are thus essential to interpreting the results. Furthermore, addition of a mRNA without a blocked 5' terminus to the reaction as in the experiment in Fig. 2 can control other nonspecific factors such as RNase activity or physical loss of the mRNA during the procedure. For the many mRNAs that contain polyadenylic acid, the demonstration of a blocked 5' end by this method, combined with the demonstration that polyadenylic acid is present at the 3' end, will be useful criteria for establishing that a mRNA has not been partially degraded during isolation.

The data presented indicate that PTH mRNA contains a blocked 5' terminus and thus is probably intact at the 5' terminus. If this is true, then the normal initiator codon *in vivo* is present and it is unlikely that the translation of PTH mRNA *in vitro* is the result of false initiation at an internal codon. This further strengthens the contention that pre-proPTH represents all of the information present in PTH mRNA that is translated into protein.

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**Fig. 2** Analysis by sodium dodecyl sulphate gel electrophoresis of the radioactive proteins synthesised in the wheat germ extracts. Translation of mRNA was as described in the legend to Fig. 1 and 5  $\mu$ l of the reactions were analysed on 15% acrylamide slab gels according to Laemmli<sup>18</sup> as described previously<sup>6</sup>. The autoradiogram was prepared by exposing the dried gel to Kodak RP Royal X-ray film for 18 h. RNA added to each reaction of 25  $\mu$ l was: *a*, 0.2  $\mu$ g haemoglobin mRNA; *b*, 0.2  $\mu$ g haemoglobin mRNA + 0.4  $\mu$ g STNV mRNA; *c*, same as *b* after treatment of the mixture with periodate and aniline; *d*, 0.4  $\mu$ g STNV RNA; *e*, none; *f*, 1.6  $\mu$ g PTH mRNA; *g*, 1.6  $\mu$ g PTH mRNA + 0.8  $\mu$ g STNV mRNA; *h*, same as *g* after treatment of the mixture with periodate and aniline; *i*, 0.8  $\mu$ g STNV mRNA. The positions of haemoglobin, STNV coat protein and pre-proPTH are indicated by H, S, and P, respectively.

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## Aqueous glasses as matrices in freeze-fracture electron microscopy

FREEZING is an important technique in the preservation of the morphology of biological materials for electron microscopic examination. Methods relying on freeze-fracturing, sometimes followed by etching, have been developed and have led to advances in the interpretation of biological ultrastructure<sup>1</sup>. Other, related applications of freezing are for the preservation of biological activity<sup>2</sup> and the preparation of biological specimens for microanalysis of low molecular weight components<sup>3</sup>. The freezing of cells and tissues can, like all other methods of fixation, give rise to artefacts, although probably to a lesser extent than the more traditional methods. The most important factors governing the quality of biological freezing include the size distribution of the ice crystals, the extent of supercooling and the possibility of osmotic dehydration and shrinkage of cells. Very high cooling rates are considered essential to minimise the deleterious effects of ice crystals, and to overcome excessive osmotic shrinkage of cells by extracellular ice formation, the use of cryoprotective agents is common. It is believed that an effective, penetrating cryoprotectant must diffuse into the cell without, at the same time, interfering with its morphology. Glycerol and dimethylsulphoxide fulfil these conditions and are widely used as cryoprotectants, although it is known that they can interfere with the physiological functions of some cells<sup>4</sup>.

The available evidence suggests that, in the appropriate conditions, the cell contents are readily supercooled<sup>5</sup>, and so it should be possible to protect cells from freeze damage by controlling the formation and growth of ice nuclei in the extracellular fluid, even in the absence of penetrating cryoprotectants. We have achieved this using water-soluble synthetic or modified natural polymers.

In favourable conditions, suitable polymers in aqueous solution can be made to yield vitreous states which are devoid of ice crystallites at low temperatures. The experimental requirements are a combination of high viscosity (that is,

**Fig. 1** 50% PVP solution quenched, freeze-fractured and subsequently etched for 5 min at 173 K. ( $\times 19,440$ )





high molecular weight and adequately high concentration) and fast cooling. Even where, for practical reasons, complete vitrification is not possible and submicroscopic ice nuclei develop during quenching, these are prevented from growing at the temperatures normally used for freeze-fracturing (120–170 K) by the very low diffusion rates in the embedding polymer solution. One of the main advantages of polymeric cryoprotectants is their inability to penetrate the cell, so they are less likely to interfere with the physiological functions, or alter the morphology. Also, by virtue of their high molecular weights, they cause hardly any osmotic flow of water from the cell.

The polymers which are most effective for the achievement of vitreous states are those with a large water-binding capacity, for example, polyvinyl pyrrolidone (PVP), polyvinyl alcohol, dextran and polyethylene glycol. Several others, such as modified starches and celluloses, although interacting strongly with water, do not yield sufficiently concentrated solutions at the molecular weights normally obtainable. Most of our exploratory work has been with PVP of average molecular weights 44,000 and 700,000, used at concentrations of 25 and 50 weight %. The aqueous solution of the polymer was quenched in melting Freon 22 and then transferred to liquid nitrogen. It was freeze-fractured (Balzer BA 360 M Freeze Etching Unit) at 173 K, some specimens subsequently being etched at this temperature. Tungsten/tantalum replicas backed with carbon were prepared and examined by transmission electron microscopy.

Figure 1 shows an electron micrograph of a quenched 50% PVP of average molecular weight 44,000. At the magnification used there is no evidence of any ice crystals, and the largest structural units are  $\sim 10$  nm in diameter. Figure 2 represents two differential scanning thermograms (Perkin Elmer DSC.2) obtained by cooling and heating the same solution. Although the fastest cooling rate which could be achieved in the calorimeter was only  $80 \text{ K min}^{-1}$ , the cooling trace shows the complete suppression of ice formation; instead a vitrification ( $T_g$ ) is indicated at 200 K. The same  $T_g$  is seen in the warming trace (10  $\text{K min}^{-1}$ ), followed by a devitrification ( $T_d$ ) at 220 K, and finally by melting ( $T_m$ ). These transition temperatures are in reasonable agreement with those reported previously<sup>8</sup>.

A 25% PVP solution, treated in the same way showed (Fig. 3) general structural features similar to those in Fig. 1, but ice nucleation sites which have given rise to spherulitic crystallites of micrometre dimensions are indicated. Raising



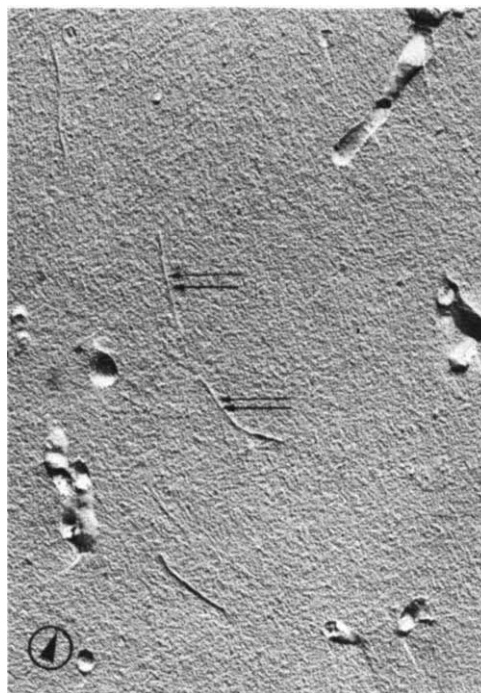
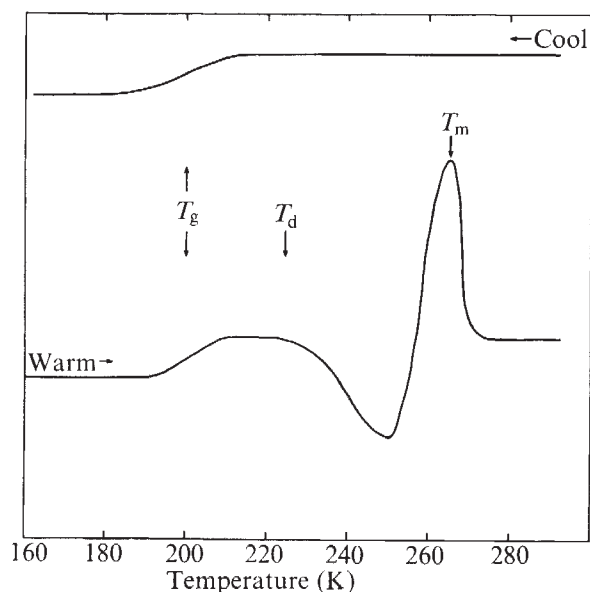
Fig. 3 25% PVP solution treated as for Fig. 1. ( $\times 19,440$ )

the temperature and maintaining it at 200 K for 2 h did not, however, lead to a further crystal growth.

These processes in the 25% solution could not be monitored by scanning calorimetry, because even the fastest cooling rate which could be achieved in the calorimeter was too slow to prevent the total freezing of the solution. This confirms a

Fig. 4 Nerve cell from the interganglionic connective from the cockroach (*Periplaneta americana*) central nervous system. Freshly dissected tissue was incubated in buffered 50% PVP for 30 min before quenching. Microtubules (arrows) can be distinguished clearly in the cytoplasmic background, showing the absence of ice damage. ( $\times 18,000$ )

Fig. 2 Differential scanning thermogram obtained by heating a 50% PVP solution.  $T_g$ , Glass transition;  $T_d$ , devitrification;  $T_m$ , melting point.





previous observation<sup>6</sup>, made using a calorimeter especially designed for much faster cooling, where it was not possible to identify  $T_g$ , because ice developed during sample cooling.

Experiments were also carried out in which PVP solutions containing single cells and tissues of animal and plant origin were similarly quenched and fractured. In all cases, extracellular medium had the appearance shown in Fig. 1 and no ice crystals could be detected in the cytoplasm. Figure 4 shows an example taken from the central nervous system of the cockroach. The cell shown comes from the centre of the tissue which is  $\sim 1$  mm thick and the absence of ice in the cytoplasm suggests that intracellular cryoprotection is afforded even when the cell is not in direct contact with the medium containing the polymer.

Further work is in progress to investigate the influence of different polymer systems and different experimental conditions on the morphological details of quenched cells and tissues. A more detailed report will be published elsewhere.

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## The hinge-bending mode in lysozyme

A COMPLETE description of an enzyme requires a knowledge of its structure and the dynamics of its function. From the crystal structures of enzymes and enzyme-inhibitor complexes and the known chemistry of model systems, it has been possible in some cases to draw reasonable inferences concerning the mechanisms of enzyme-catalysed reactions. Little has been done so far, however, to supplement such essentially static results by an investigation of the reaction dynamics. This requires an understanding of the internal motions of the enzyme, as well as those of the substrate, since both are likely to be essential to the function. Here we present a theoretical study of a low frequency vibration involving the two globular lobes of lysozyme between which the cleft containing the active site is located<sup>1</sup>. Any motion involving this cleft could play a part in the catalytic activity; in fact, atom displacements of up to 0.75 Å found in a comparison of the free enzyme and the enzyme-inhibitor complex indicate that the cleft has closed down in the latter<sup>2</sup>. The force constant for the low frequency bending vibration corresponding to the opening and closing of the cleft is obtained from empirical energy functions<sup>3</sup>. Because the protein surface moves appreciably during the vibration, damping effects resulting from the viscous dissipation in the solvent are included in the calculation<sup>4,5</sup>.

To model the hinge-bending mode, the lysozyme molecule is assumed to be composed of two essentially rigid lobes (lobe 1: residues 5–36 and 98–129; lobe 2: residues 40–94), connected by

a hinge region consisting of the amino-terminal end (residues 1–4) and two short polypeptide chain segments (residues 37–39, 95–97) running from one lobe to the other<sup>1</sup>. A natural axis parallel to the cleft is obtained for the bending motion by passing a line through C<sub>α</sub>38 and C<sub>α</sub>97, an excellent view of the cleft and the chosen axis can be obtained from diagram L4 of Dickerson and Geis<sup>6</sup>.

The bending motion of lysozyme in the presence of solvent is determined by the Langevin equation for a damped harmonic oscillator<sup>7</sup>, which has the form

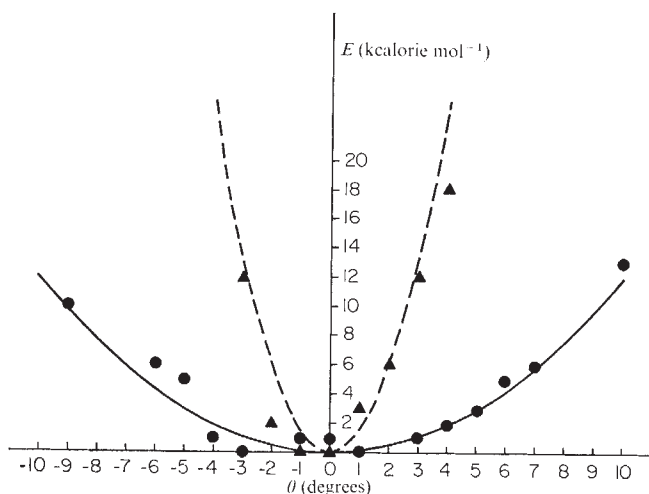
$$I \frac{d^2\theta}{dt^2} + f \frac{d\theta}{dt} + k\theta = N_r(t) \quad (1)$$

Here,  $\theta$  is the angular displacement from the equilibrium position,  $I$  the effective moment of inertia,  $f$  the friction coefficient, and  $k$  the force constant associated with the relative motion of the lobes;  $N_r(t)$  represents a randomly fluctuating torque characteristic of Brownian motion. The properties of a system governed by equation (1) are well known<sup>7</sup>. To apply the results to the present case, it is necessary only to determine the parameters  $I$ ,  $f$ , and  $k$ .

The force constant  $k$  was estimated from an empirical energy function that expresses the total conformational energy as a function of the Cartesian coordinates of the heavy atoms<sup>8</sup>; it includes terms associated with bond lengths, bond angles, dihedral angles, hydrogen bonds and non-bonded interactions. To obtain an equilibrium starting structure, the real space refined coordinates of lysozyme<sup>8</sup> were subjected to 200 steps of steepest-descent energy refinement. The potential function for bending was then determined by rotating residues 39–96 rigidly about the chosen axis and computing the conformational energy of the distorted protein as a function of the bending angle. To obtain a more realistic potential function, the same rigid rotations were performed, but at each bending angle there followed a 100-cycle energy minimisation which allowed for adiabatic relaxation of the protein<sup>9</sup>. The bending angle was kept fixed during the relaxation by imposing constraints on residues distant from the hinge in the interior of each lobe (residues 44–51, 61 and 65–73 in lobe 2, residues 109–122 in lobe 1). The results of the two calculations are shown in Fig. 1; a parabolic fit to the points yields the force constants  $k_r = 4.1 \times 10^{14}$  erg rad<sup>-2</sup> mol<sup>-1</sup> (rigid), and  $k_a = 3.3 \times 10^{13}$  erg rad<sup>-2</sup> mol<sup>-1</sup> (adiabatic). Total moments of inertia about the bending axis were computed for residues 38–97 ( $I_2$ ) and for the rest of the molecule ( $I_1$ ); the values are  $I_2 = 1.01 \times 10^{-10}$  g cm<sup>2</sup> mol<sup>-1</sup> and  $I_1 = 1.08 \times 10^{-10}$  g cm<sup>2</sup> mol<sup>-1</sup>. Water molecules associated with the protein were ignored in this calculation. The friction coefficient was estimated by a modified Stokes law<sup>9</sup> appropriate for the hydrodynamic interaction of two spheres of radius 10 Å separated by a gap of 5 Å, undergoing small relative motions about an axis 20 Å from the sphere centres; the resulting value is  $f = 5.7 \times 10^2$  g cm<sup>2</sup> s<sup>-1</sup> mol<sup>-1</sup>.

If hydrodynamic effects are neglected, equation (1) reduces to the equation for a simple harmonic oscillator. With the approximation  $I = (I_1 I_2 / I_1 + I_2)$ , the resulting frequencies are  $\omega_r = 15$  cm<sup>-1</sup> and  $\omega_a = 4.2$  cm<sup>-1</sup>. These very low frequencies result from a balance between the large force constant and the large moment of inertia associated with the bending motion. The effective CCN bending force constant for an  $\alpha$  carbon is  $2 \times 10^{12}$  erg rad<sup>-2</sup> mol<sup>-1</sup>, less than 1/10 of  $k_a$ . The additional contributions to  $k_a$  come from a large number of non-bonded contacts (20–50, depending on the bending angle), involving atoms in the hinge region and in the lobes (for example, for closing, Leu 56 O... Trp 108 N<sub>ε1</sub>, Trp 63 C<sub>η2</sub>... Asp 101 O<sub>δ2</sub> and for opening His 15 N<sub>ε2</sub>... Thr 89 O<sub>γ1</sub>, His 15 C<sub>δ2</sub>... Thr 89 O<sub>γ1</sub>). The large difference between  $k_r$  and  $k_a$  arises from small atomic displacements ( $\leq 0.5$  Å) that alleviate the close non-bonded contacts produced by the rigid motion.

Introduction of the hydrodynamic terms in equation (1) demonstrates that for an aqueous medium the inequality



**Fig. 1** Change in conformational energy produced by opening ( $\theta < 0$ ) or closing ( $\theta > 0$ ) the lysozyme cleft. Calculated values are for the rigid bending potential ( $\blacktriangle$ ), and for the adiabatic bending potential ( $\bullet$ ); the curves are parabolic fits to the points. The  $\theta = 0$  value corresponds to the minimum energy geometry for each of the potentials; the adiabatic minimum corresponds to an energy 34 kcalorie mol<sup>-1</sup> below the rigid curve and is displaced toward a more open configuration by 5°.

( $f^2 > 4Ik$ ) is satisfied so that the motion is 'overdamped'; that is, rather than vibrating periodically with the calculated frequency, the two lobes move relative to each other in a diffusive manner in accord with the Smoluchowski equation<sup>7</sup>. Quantitatively, the inertial effects decay rapidly, with a time constant  $\tau_1 = (I/f) \approx 10^{-13}$  s, and the relaxation time for displacements from equilibrium is  $\tau_0 \approx f/k_a \approx 2 \times 10^{-11}$  s; this latter value is to be compared with the undamped vibration period,  $\tau = 8 \times 10^{-12}$  s.

The time constant  $\tau_0$  for the diffusional displacement of the lobes is sufficiently large to justify the use of the adiabatic potential; that is, the local torsional and bending motions required to remove the repulsive contacts have vibrational periods that are much shorter than  $\tau_0$ .

It is possible that the cleft motion involving the active site cleft has a significant effect on the rate constants for substrate binding to lysozyme. The time required for a molecule the size of glucose to diffuse freely 5 Å is  $> 5 \times 10^{-10}$  s at 300 K, and, thus, substantially  $> \tau_0$ . Since opening or closing fluctuations on the order of twice the root mean square value ( $\langle \theta^2 \rangle^{1/2} \approx 1.6^\circ$ ) correspond to displacements of over 1 Å in the outer part of the cleft region, the coupling between substrate binding and the cleft vibrational mode requires a more detailed study taking into account the enzyme-substrate interaction; for example, the motion could be significantly perturbed by the presence of the substrate.

It is important also to note that the lobe motion examined here is so rapid, compared with the transport phenomena usually considered for biopolymers (such as diffusion and sedimentation), that the traditional approximations of incompressible and quasi-steady flow used in determining  $f$  have to be questioned. In particular, the Reynolds number, which measures the deviation from quasi-steady flow<sup>10</sup>,  $= R^2/\eta\tau_0$ , is 0.1–0.2 ( $R$  is the lysozyme radius and  $\eta$  is the kinematic viscosity) and the Mach number, which measures the deviation from incompressible flow<sup>11</sup>,  $= R/c\tau_0$ , is 0.1 ( $c$  is the speed of sound in water). Thus, the present treatment is valid only as a first approximation. Moreover, a more detailed molecular approach to the solvent behaviour (for example, the water in the cleft) might be appropriate, although computer studies indicate that bulk water responds in a viscous manner to

shearing forces on the molecular scale for times greater than  $5 \times 10^{-13}$  s (ref. 12).

The method used and the results obtained for the diffusive behaviour of the hinge-bending mode of lysozyme may be of wider interest in the theory of biopolymers. Active site clefts are found in many enzymes<sup>13</sup> and their opening and closing may be generally involved in the substrate-binding step. Also, the relative displacements of domains or subunits, which are linked by flexible hinges (as in the immunoglobulins<sup>14–16</sup>) or interact only by non-bonded forces (as in the haemoglobin tetramer<sup>17</sup>) are likely to require similar considerations, in which the balance between internal forces and hydrodynamic damping effects are essential. Finally, any experiments designed to observe such motions<sup>18,19</sup> will have to take account of the results obtained here (for example, preliminary calculations indicate that the Raman band associated with the lysozyme bending mode will be damped out in solution, although binding of an inhibitor could lead to underdamped motion and an observable Raman band).

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## Structure of bilirubin

THE biological breakdown of haem proceeds through a series of intermediates of which the orange-yellow bile pigment, bilirubin, the chromophore responsible for coloration in the various forms of jaundice, is a key member. In the adult human, for example, erythrocytes have a lifetime of about 3 months and their destruction produces about 300 mg bilirubin each day<sup>1,2</sup>. Although there were some early doubts (arising from the possibility of a cyclisation between the C18 vinyl group and the oxygen function at C19)<sup>3</sup>, the gross chemical structure (Fig. 1) for bilirubin has been beyond reasonable dispute for

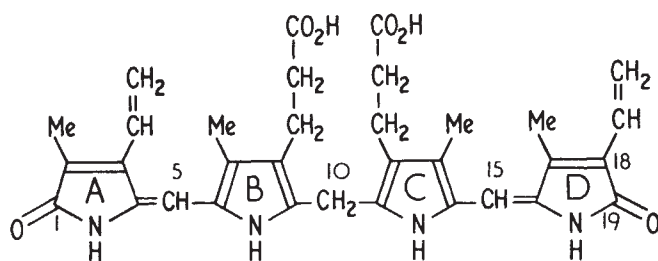


Fig. 1 Gross structure of bilirubin (also called bilirubin IX $\alpha$ ).

many years<sup>4</sup>. There remain, however, considerable structural uncertainties with respect to (1) stereochemistry, the geometry around bridges 5 and 15 being unknown, and represented variously as *E* or *Z* in the current literature. (The two configurations, *E* and *Z*, have been recognised in a model pyrromethanone system, however, (see ref. 5 and Fig. 2)); (2) tautomerism, although spectroscopic evidence favours the lactam formula for rings A and D (shown in Fig. 1) over the lactim<sup>6-9</sup>; and (3)

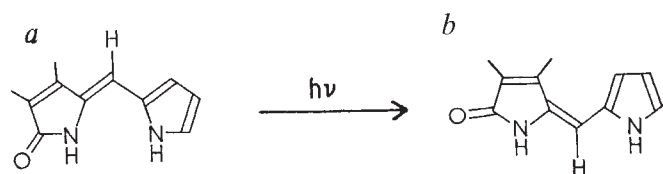


Fig. 2 a, *Z* configuration; b, *E* configuration.

conformation, although several speculations, supported by spectroscopic evidence and model making, are available<sup>10-17</sup>. We report the X-ray analysis of bilirubin—the first such analysis of a naturally occurring linear tetrapyrrole—which shows that bilirubin has the *Z* configuration at the C4–C5 and C15–C16 bonds, and possesses in the crystal a ridge tile conformation with considerable intramolecular hydrogen bonding.

Crystals of bilirubin were grown by allowing diethyl ether to diffuse slowly (isothermal distillation technique, room temper-

Fig. 3 Structure of bilirubin. Hydrogen bonds are indicated by fine lines.

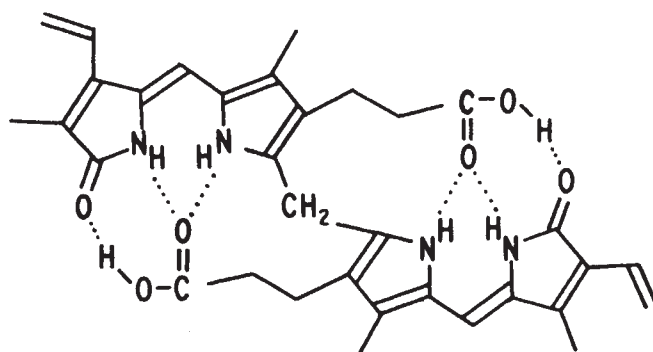
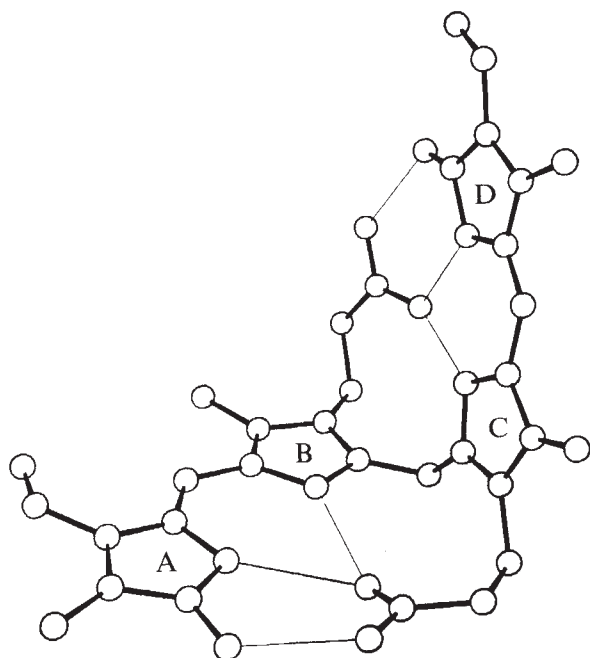


Fig. 4 Structure shown in Fig. 3 in simplified form.

ature, dark) into a solution of bilirubin in anhydrous pyridine containing a trace of anhydrous ammonia. Crystals were mounted in glass capillaries in the presence of mother liquor. Crystal data:  $C_{33}H_{36}N_4O_6$ , molecular weight 584; crystal class triclinic; space group  $P\bar{1}$ ;  $a = 19.439$ ,  $b = 11.707$ ,  $c = 15.500$  Å;  $\alpha = 97.19^\circ$ ,  $\beta = 100.22^\circ$ ,  $\gamma = 118.20^\circ$ ;  $V = 2,969$  Å<sup>3</sup>  $\rho_{\text{calc}} = 1.31$  g cm<sup>-3</sup> for  $Z = 4$ .

X-ray diffraction patterns recorded from these crystals generally revealed that reflections with odd *h* indices were diffuse, indicating disorder in the *x* direction. (It seems likely that this disorder may have frustrated previous attempts to determine the structure.) The amount of this diffuse scatter varied from crystal to crystal: in some cases the *h*-odd reflections were so diffuse that the triclinic cell seemed to be halved ( $a = 9.720$  Å,  $Z = 2$ ). In the crystal selected for detailed examination, the *h*-odd reflections had initially been somewhat diffuse, but had gradually sharpened during maturation (5 weeks, room temperature, dark). The diffraction pattern then contained virtually no diffuse effects.

Two complete sets of data were collected from this crystal: a set of photographic Weissenberg data collected about two axes ([010] and [111]) and scanned using the SRC Microdensitometer Service at Harwell; and a set of counter-data collected using a Nonius CAD4 diffractometer. The photographic set contained 1,323 unique observed reflections (37% of the possible number of unique reflections to a limit of  $2\theta(\text{CuK}\alpha) = 80^\circ$ ); and, of the 6,095 reflections measured by the diffractometer, only 1,408 obeyed the condition  $F_{\text{obs}} > 3\sigma F_{\text{obs}}$ . This serious lack of data arose because the average intensity of the X-ray reflections rapidly decreased with increasing scattering angle: few reflections were found above  $2\theta(\text{CuK}\alpha) = 70^\circ$ .

The structure was solved using the diffractometer data and a computer program written by Dr G. M. Sheldrick (Cambridge), which uses a multisolution procedure incorporating negative quartets. Details of this solution will be published elsewhere. Full matrix constrained-bond length refinement of the photographic data (which proved the better set for refinement purposes) with hydrogen atoms in geometrically calculated positions has given  $R = 12.5\%$ . Because of the small number of observed reflections the structure is not an accurately

Fig. 5 Model compound 3.

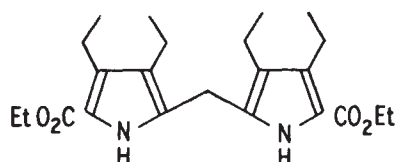
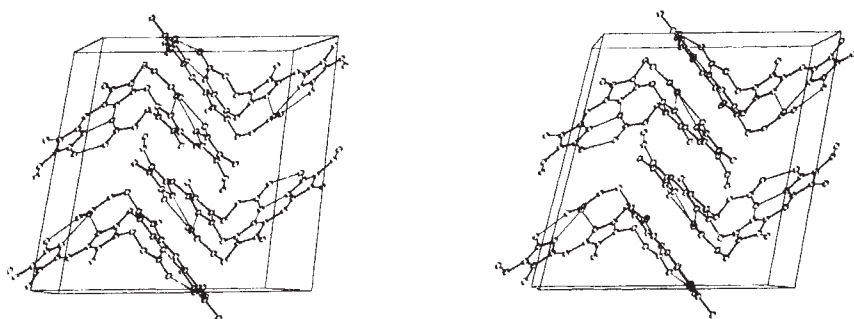




Fig. 6 Packing of bilirubin molecules in the unit cell. The *b* axis is approximately perpendicular to the page, and the *c* axis is horizontal.



determined one: estimated standard deviations of individual bond lengths were generally in the range 0.02–0.05 Å.

The two independent molecules of bilirubin in the asymmetric unit of the structure both have the conformation shown in Fig. 3 and represented in a simplified way in Fig. 4. The molecule takes the form of a ridge tile, the ridge being along the line C8<sup>1</sup>–C10–C12<sup>1</sup>. Thus rings A and B lie in one plane, and rings C and D in another, the interplanar angle being about 98°. Six intramolecular hydrogen bonds (two sets, each involving a carboxylic acid group, a pyrrole-imino hydrogen, and a terminal lactam system) stabilise this conformation. The average N...O and O...O distances in these hydrogen bonds are 2.70 and 2.53 Å, respectively. There is no evidence for intermolecular hydrogen bonds: the low solubility of bilirubin in water, which has important biological consequences, can readily be understood in terms of this pattern of hydrogen bonding. Although the hydrogen bonding, and therefore the conformation, may well be altered in very polar solvents (such as dimethylformamide and methanolic ammonia), it seems likely that in less polar solvents (such as chloroform) the conformation with six hydrogen bonds (Fig. 3) will be retained. Unfortunately, the X-ray data are neither sufficiently numerous nor sufficiently accurate to allow determination of the position of the hydrogen atoms in these hydrogen bonds and we are not able to make a clear cut assignment of either the lactam or the lactim formulations.

The X-ray result provides independent proof of the correctness of the gross chemical structure assigned to bilirubin by Fischer *et al.*<sup>4</sup> The stereochemistry about the double bonds at the *meso*-bridges (C5 and C15) is frequently, even in modern work<sup>18,19</sup>, represented as *E*. The *Z* configuration (Fig. 4) is now shown to be correct. This type of structure seems to have been first portrayed by Blauer and King<sup>14</sup>; the first discussion in detail was by Kuenzle *et al.*<sup>15</sup>.

The observed bond lengths suggest that delocalisation over an individual conjugated system of two pyrrole rings (that is, A+B or C+D) is rather limited since C4–C5 and C15–C16 seem to be essentially double bonds (average 1.30 Å) whereas C5–C6 and C14–C15 resemble single bonds (average 1.48 Å). This supports the view that bilirubin can be regarded as a 2,2'-dipyrrolylmethane (that is, rings B | C) with conjugating substituents at the  $\alpha$  positions (compare with the model compound shown in Fig. 5)<sup>18</sup>.

The packing of the four bilirubin molecules in the unit cell is shown in Fig. 6. Taking the original analogy further, we now have a stack of ridge tiles interleaved with a similar but inverted stack. Figure 6 suggests an explanation for the diffuse *h*-odd reflections observed in the diffraction patterns of most of our crystals: enantiomeric conformations of bilirubin (related by inversion centres) have very similar ridge tile shapes and the orientation of any ridge tile is virtually unaltered by a rotation of 180° about a direction approximately parallel to the *a*\* axis (vertical in Fig. 6). On the basis of Fig. 6, it is not difficult to imagine disordered structures which would give an apparent halving of the *a* axis. We have, indeed, collected a third set of data from a disordered crystal, and successfully refined it to *R* = 19.7% by assuming that either enantiomer may occupy any molecular site, the packing of the ridge tiles remaining constant.

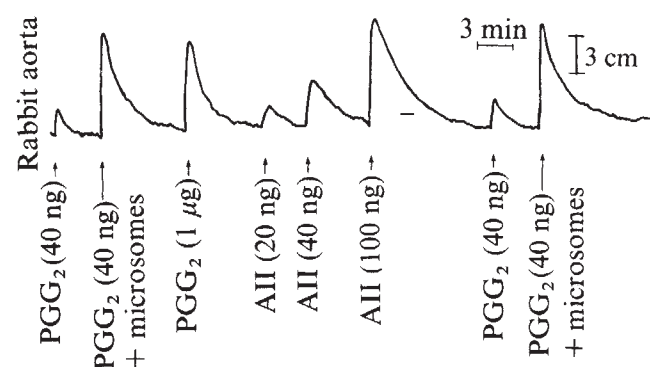
We thank Dr S. Neidle and Dr J. C. M. Stewart for an earlier attempt on this problem; Dr G. M. Sheldrick (Cambridge) for assistance; Dr M. Elder and his staff at the SRC Microdensitometer Service for measuring the Weissenberg films; and the MRC for financial support.

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## Erratum

In the article "Identification of an enzyme in platelet microsomes which generates thromboxane A<sub>2</sub> from prostaglandin endoperoxides" by P. Needleman, S. Moncada, S. Bunting, J. R. Vane, M. Hamberg and B. Samuelsson (*Nature*, 261, 558; 1976) microsomes were added to the wrong dose in Fig. 5 and the vertical scale (3 cm) was too large. Both are correct in the version reprinted below.

# matters arising

## Deposit-forming by zinc dithiophosphates in base oils during oxidation

A RECENT report by Bird and Galvin<sup>1</sup> has described experiments in which specimens of En 31 steel were immersed in solutions of zinc dialkyldithiophosphates in white oil and either heated over a range of temperatures for 16 h or rubbed for 10 min against a disk of the same material. Electron spectroscopic investigations of the surface films formed at 140 °C showed a product having a zinc-phosphorus-sulphur ratio of 1:1:0.6, and this ratio was said to be constant over a range of conditions, and was thus assumed to be a decomposition product of the original additive. Zinc phosphate, sulphide and sulphite formations were not observed.

Work in our laboratories on the oxidation stabilities of mineral oil blends of zinc dithiophosphates has revealed similar decomposition residues. The formation of these was, however, found to depend on both the hydrocarbon group in the dithiophosphate and the nature of the base oil. Thus, when a solution of a zinc dissecondary alkyl dithiophosphate in a high sulphur content base oil was heated at 120 °C for 164 h and subjected to a stream of dry oxygen, a residue was formed which had a zinc (24.6%)-phosphorus (12.6%)-sulphur (4.5%) ratio of 1.0:1.08:0.37, which was not too dissimilar from that found at the iron surface by Bird and Galvin<sup>1</sup>. Further, X-ray examination of this residue showed that it was a complex inorganic material in which zinc oxide, sulphide or phosphate was absent. When blends of this additive in low sulphur content mineral oil base fluids were similarly tested this residue was not produced.

Substitution of zinc diprimary alkyl or diaryl dithiophosphates in the above range of base oils failed to produce any residues. It was therefore concluded that the decomposition was a function both of the base oil and the hydrocarbon group in the dithiophosphate. Previous work has suggested<sup>2</sup> that the decomposition of zinc dialkyldithiophosphates is dependent on the number of  $\beta$ -hydrogen atoms attached to the alkyl constituent, and this is fully consistent with the results obtained in our present work.

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## Haemoglobin type and superovulation in ewes

PANT and Pandey<sup>1</sup> reported that haemoglobin (Hb) type in Binaneri ewes influences the ovulatory response to exogenous gonadotropin (pregnant mare's serum gonadotropin, PMSG). The practical and physiological implications of this finding prompt us to present the results of a study of the relationship between Hb type and ovulatory response to PMSG in parous, non-lactating Welsh Mountain ewes.

Eighty-six ewes were given 1,000, 1,100 or 1,250 IU PMSG (Folligon, Intervet) on day 12 (day 0 is the day of oestrus) of the oestrous cycle and ovulation rate assessed by counting the corpora lutea present at laparotomy on days 2–8 of the subsequent cycle. Hb type was determined by starch gel electrophoresis in a Tris-EDTA borate buffer system, pH 8.2 (ref. 2). An analysis of variance of ovulation rate, after transformation of the raw data to  $(x + 1)^{1/2}$ , showed no significant effect of either Hb type or dose of PMSG. Table 1 shows the mean ovulation rates for Hb type, the data being pooled for dose of PMSG.

A further 16 ewes were given 500 IU PMSG and ovulation rate examined as before. Mean  $\pm$  s.e. (number of ewes) ovulation rate for AA, BB and AB Hb types were  $1.3 \pm 0.21$  ( $n = 6$ ),  $1.6 \pm 0.24$  ( $n = 5$ ) and  $2.2 \pm 0.37$  ( $n = 5$ ), respectively.

These results argue strongly against a correlation between Hb type and ovulatory response to superovulation in Welsh Mountain ewes. Breed or environmental differences may, however, explain the discrepancy between our results and those of Pant and Pandey<sup>1</sup>. It is clear that Hb type in Welsh Mountain ewes is not a major determinant of the very substantial individual variation in ovulation rate observed after administration of PMSG.

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<sup>1</sup> Pant H. C., and Pandey, M. D., *Nature*, **258**, 738–739 (1975).

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## Volcanic triggering of glaciation

IN his latest contribution to the discussion of the links between volcanism and glaciation<sup>1</sup>, Bray perpetuates a concept that he proposed in much stronger terms in his two earlier contributions on the same subject<sup>2,3</sup>; namely that there is evidence of "an apparent correlation between major phases of global ice advance and ash eruptions in New Zealand, Japan and southern South America over the past 42,000 yr". This represents a slight modification of his earlier view that there were worldwide 'waves' of volcanic activity that could be correlated with the same ice advances<sup>3</sup>.

I am not in a position to comment on the evidence from Japan or New Zealand, but in my opinion the postulation of correlatable episodes of volcanic activity and ice advance in southern South America is extremely unwise, simply because there is so little factual evidence. All such postulations are based on the widely quoted but rarely read work of Auer<sup>4–7</sup>. It is clear from his descriptions that the ash horizons that Auer used in his work, were, in most cases, air-fall ashes from single volcanic eruptions, which may have been quite small. There is no evidence for any massive eruptions, nor for any waves of eruptions. The weakness of the evidence for such waves is compounded by the very small number of published radiocarbon dates; there are probably less than a dozen such dates for volcanic eruptions in the whole of South America.

It is quite correct, as Bray states, that "the possibility that large Pleistocene

Table 1 Hb type and ovulatory response to PMSG\* in Welsh Mountain ewes

| Hb type                          | AA             | BB             | AB              | Total          |
|----------------------------------|----------------|----------------|-----------------|----------------|
| Ovulation rate (mean $\pm$ s.e.) | $8.6 \pm 0.96$ | $8.2 \pm 0.90$ | $10.0 \pm 1.29$ | $8.9 \pm 0.63$ |
| No. of ewes                      | 20             | 35             | 31              | 86             |

\*PMSG at 1,000–1,250 IU.



ice sheets were initially triggered by massive volcanic eruptions is not contradicted by the geological record". But until further data are available from Patagonia, and especially studies of the ash deposits by volcanologists, the evidence from southern South America should not be used either to support or contradict the argument.

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- <sup>1</sup> Bray, J. R., *Nature*, **260**, 414–415 (1976).
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BRAY REPLIES—The main point of Francis<sup>1</sup> is that the evidence of late Pleistocene and Holocene volcanism in southern South America<sup>2</sup> is based on a small number of published radiocarbon dates. The reason I originally included the southern South America volcanic data was that both the radiocarbon-dated ashes and the indirectly dated ashes all occurred at the same time as periods of volcanic activity in New Zealand and Japan. This occurrence seemed to be more than coincidental and I suggested that the apparent synchronicity of volcanic ash data from the three areas reinforced the hypothesis that there have been global waves of volcanic activity separated by periods of relative quiescence. Since then, South American volcanic ash dates of 9,000, 11,000, 21,000, 22,000–26,000 and 30,000 yr BP have been published<sup>3</sup> which are also coincident with the New Zealand and Japanese volcanic phases. In addition, I have made a preliminary compilation of North American and European volcanic ash dates which show the following distribution (in 1,000 <sup>14</sup>C yr b.p.): 0.2–0.4; 0.6–0.8; 1.2–1.3; 1.6–1.8; 2.0–2.6; 2.8; 3.3–3.5; 3.9; 4.4; 5.0; 6.6; 8.3–8.9; 11.3; 11.7–12.0; 13.1; 18.6; 19.7; 28.5; 31.0; 35.0; 39.5–40.5. A comparison of these dates with the New Zealand and Japanese data in Table 1 (ref. 2) shows that all but five dates are synchronous.

Francis' other point, that some of the southern South American eruptions were minor is correct, but not contradictory to the theme of global waves of volcanic eruptions in the late Pleistocene which have influenced climate and perhaps triggered glacial expansion. In any case, if the large mid-continental Northern Hemisphere ice sheets were triggered by massive eruptions<sup>4</sup>, then it is likely that these eruptions occurred in the Northern Hemisphere or perhaps the tropical portion of the Southern Hemisphere. It is in these regions that

many of the largest Pleistocene eruptions have occurred as shown by the land record and especially by the ash layers in the ocean<sup>5</sup>.

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## Acoustic Bragg diffraction from human tissues

NICHOLAS AND HILL<sup>1</sup> claim to have presented "qualitative evidence that the method can provide objective characterisation of tissue structure", and that the "diffraction phenomenon . . . can be quantitatively related to the specific structure of the type of tissue".

They present their evidence for the objective characterisation in Fig. 4, where three sets of tissue specimens (of unspecified condition) have a statistical significant difference over a portion of the curve, the curve having been obtained by an arbitrary seven-point smoothing. Whereas the first claim of the authors may be true, the second, regarding the specific structure of the type of tissue, is surely not substantiated by the evidence presented. Differentiation between tissue types may have been demonstrated but there is no evidence presented for its relationship to specific structure. Indeed, it is known<sup>2,3</sup> that the Fourier transform of angular correlation is a spatial spectrum, so that the peaks in the spectrum of Nicholas and Hill's Fig. 3 indicate distances of significance in the tissue specimens, presumably related to structure, as the parallel with X-ray diffraction suggests. This potential source of helpful fundamental information has been removed by cavalier smoothing.

In a field characterised by complexity<sup>4</sup>, bedevilled by incomplete reporting<sup>5</sup>, and yearning for independent confirmation, the significance of the results reported<sup>1</sup> demands more careful and precise consideration.

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- <sup>1</sup> Nicholas, D., and Hill, C. R., *Nature*, **257**, 305–306 (1975).
- <sup>2</sup> Welsby, V. G., *J. Sound Vib.*, **8**, 64 (1968).
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NICHOLAS AND HILL REPLY—We accept the criticism<sup>1</sup> of our statement<sup>2</sup> that "this diffraction phenomenon . . . can be quantitatively related to the specific structure of the type of tissue". This statement is out of place in the article

concerned and should have been omitted: its truth, however, has been confirmed in other publications<sup>3,4</sup>. The other points raised by Chivers are, in our view, unjustified and in some cases inaccurate.

In all of our investigations the tissues were freshly excised, pathologically normal specimens: although this was not stated explicitly it was thought that the implication of comparing pathologically abnormal tissues with the reported results, justified such an omission.

Chivers' criticism<sup>1</sup> of the curves depicted in our original Fig. 4 (ref. 2)—that the range of the seven-point average was not specified—is, we believe, taken in context, not valid. It was stated in our report<sup>2</sup> that the power spectra were calculated using a fast Fourier transform and, as such, they are both finite and discrete. We also stated that the data in each resulting spectrum was averaged over consecutive seven-points. Thus it is that the range of our averaging was over the whole spectrum, as should be obvious if Fig. 4 is compared with Fig. 3.

Finally, Chivers' comments on the validity of our smoothing procedure should be considered in the light of our objective. We clearly stated that "several possible analytical approaches could be taken towards establishing the significance of differences or similarities between such traces and we report here the results of one of these". Useful information has indeed been removed by smoothing, but the advantage gained is the relative ease with which different traces can thus be compared. It is apparent from our Fig. 3 that the peak separation is of the order of 0.01 cycles per degree, which corresponds to a separation in the angular traces of Fig. 2 of approximately 100°; hardly a significant contribution when considering the 50° of trace illustrated. Chivers' comments on this point, although strictly valid, are therefore of limited relevance to the results presented. We did not imply that the peaks in the power spectrum were artefacts, but merely indicated that the picket-fence effect must have led to some degradation of the resulting power spectra.

Our article was intended purely as an introduction to a new and exciting aspect of tissue characterisation and did not claim to be a complete report. It remains our belief, however, that the results we presented are both significant and precisely defined.

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# reviews

## Sorting the muddle in the Middle

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*After the Australopithecines: Stratigraphy, Ecology, and Culture Change in the Middle Pleistocene.* (World Anthropology.) Edited by Karl W. Butzer and Glynn Ll. Isaac. Pp. xv+911. (Mouton: The Hague and Paris, 1975.) (Distributed in US and Canada by Aldine: Chicago.) Dutch Guilders 127.50.

THIS is a most timely review of work on the span of the Quaternary, dubbed broadly Middle Pleistocene, younger than the australopithecine-bearing strata that have been calibrated by potassium-argon dating. The period is terminated by the advent of the Later Pleistocene which is followed by the lower limit of radiocarbon dating at  $\pm 50,000$  yr b.p. The continental deposits in 'the middle' represent a period when reliable dates from sources independent of the fossil faunas or artefact assemblages for which an age is sought are extremely scarce. Lithological comparisons are rendered difficult by isolation of the sedimentary basins and the parochial nature of the deposits. Attempts have been made to use climatic change as the vehicle for correlation, but these are largely unsatisfactory until the Last Interglacial (Ipswichian—Eemian) denotes the onset of the Later Pleistocene.

Middle Pleistocene continental stratigraphy in the Old World is therefore a muddle which this volume does a great deal to clarify by isolating the problems and suggesting solutions. I must carp a little at the length, not to say verbosity, of several of the contributions. Thus 21 papers amount to 887 pages, including excellent black and white illustrations. This yields an average of 42 pages per contributor and is the reason that for most students of the Quaternary, from the undergraduate to professorial grade, this volume priced at £26 sterling per copy, sadly seems destined almost solely for library shelves.

With this caveat it must be noted that the papers have been carefully selected by the editors and the scope of the book is broad but balanced. The geological contributions include the record of deep sea cores (Shackleton), the Mediterranean littoral (Butzer), volcanics of the Massif Central (Bout), European Loess stratigraphy (Kukla) and sediments of East Anglia (Glad-

felter). On this substratum, interglacial correlation is discussed by Turner, and various aspects of mammalian palaeontology by Kahlke, Janossy, Jaeger and Maglio, respectively.

The lithic industries of Africa, the Middle East and Europe are then placed into the stratigraphic framework by Mary Leakey, Isaac, Deacon, Bar-Yosef, Clark, Freeman and de Lumley. Finally, Pilbeam reviews the fossil evidence for Middle Pleistocene tool-making hominids.

The volume is an essential reference book for all workers involved in the problems of the Middle Pleistocene. Space does not permit an analysis of the papers beyond noting that they provide the most up-to-date statement in this field. The papers were revised and submitted to the editors after a conference organised in July 1973 by the Wenner-Gren Foundation for Anthropological Research at Burg Wartenstein, Austria.

The meat of the conference and the conclusions arising from this meeting between workers in different disciplines are provided in two short concluding chapters by Butzer on the Geology and Ecology (18pp) and Isaac on the Archaeology and Anthropology (14pp), together with two Appendices containing correlation charts and the recommendations of the meeting.

The most potent wine is kept until the end of the book (pp901–903). It was recommended unanimously by the conference that the lower boundary of the Middle Pleistocene be defined as

the junction between the Matuyama Reversed Epoch and the Brunhes Normal Epoch of the palaeomagnetic reversal chronology (that is at about 0.7 Myr). Magnetostratigraphy is still at an early stage of development but the reasons for choosing "this unique global criterion" include: the difficulty experienced in trying to apply other methods; its application to both marine and continental sediments; the fact that it can be established in many existing litho-stratigraphic units; and in some areas there is a good fit with the existing local placement of the boundary. The fact that a major change from the current location of the boundary would be necessary in some other areas points the need to use some global parameter.

The proposed upper boundary is the beginning of the marine transgression associated with the onset of the Last Interglacial. Although the inadequacy of this boundary is recognised it seems to be the best available at present. It is to be hoped that those concerned with codes of stratigraphic nomenclature will recognise the value of the proposed palaeomagnetic reversal boundary for the lower limit and that stratigraphic connoisseurs will find this new wine acceptable to the international palate. □

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## Controversial morphometrics

*Uniqueness and Diversity in Human Evolution: Morphometric Studies of Australopithecines.* By Charles E. Oxnard. Pp. viii+133. (University of Chicago: Chicago and London, 1975.) £9.00.

IN spite of caveats about the tentative nature of the results in this volume Oxnard has thrown down the gauntlet firmly at the feet of hominid paleontologists. Implicitly throughout much of the book, and explicitly in the final chapter, he claims that the Australopithecines (including *Homo habilis* and '*Homo africanus*') should be formally

excluded from any ancestral relationship to man. The basis for this view is that morphometric studies he cites show them not only to be unique but also, in several computer analyses their nearest analogues are orang-utans, specialised Asian apes. The test is whether Oxnard's book contains sufficiently cogent evidence for such a bold view.

The book is in three sections. The first two chapters consist of a theoretical discussion about how best structure and behaviour can be equated, a review of discriminant analysis and methods for displaying the results, and a review of locomotor classification.

The sine-cosine graphs, apart from inducing vertigo, do provide a convenient way of detecting specimens which although projected on the main canonical axes, in morphometric 'reality' lie some distance away. These chapters are heavy going and the writing style, with long and involved sentences, makes the reader's task more difficult.

The middle section is the longest and reviews multivariate morphometric studies on fossil hominid remains of the upper and lower limb girdles, several tali, humeral fragments, metacarpals and a toe bone. This is no place to take issue on technical details of the analyses and Oxnard fairly draws attention to the differences between the way he and other workers interpret the results of canonical analysis.

The last section is an attempt to synthesise the results followed by a discussion of their relevance, and it is here that the greatest controversy lies. It has recently become evident that locomotor assessments of early hominid fossils were bedevilled because modern human bipedalism was considered as the only bipedal arrangement possible: no close affinities to modern bones, no bipedalism. Now it is widely recognised that other models may have existed either as precursors of the modern human system or as parallel models which were destined for extinction. Therefore the 'uniqueness' of some of the fossils comes as no surprise.

Some of the analyses are claimed to show similarities between fossil hominid fragments and orang-utan bones,

implying a close functional rather than a genetic relationship. Whereas the statistical implications of these results are exhaustively examined their conversion back into any biological reality is naive. Most of the fossils are fragmentary, and some form only part of functional units of which other parts are preserved. There is no examination of which variables underly the association with the orang-utan, nor is there any attempt at a functional anatomical or biomechanical explanation of why bones that do not look alike behave similarly in the analyses.

Morphometrics is only one of the methods available to hominid paleontologists in their attempts to analyse the functional implications of hominid fossils and unravel the phylogeny of early man. Such studies were at one time properly criticised for eschewing numerical data, but such data taken in isolation do not guarantee relevant results. The evidence presented in this book, based as it is on only a fraction of the available fossil evidence, is too narrow and conceptually limited to play more than a subsidiary role in the very necessary re-evaluation of our ideas about early hominid structure, function and phylogeny.

Oxnard has nonetheless performed a useful service; the controversy produced by this book may well stimulate the necessary, more balanced, research.

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## Mapping the insect brain

*Atlas of an Insect Brain.* By Nicholas J. Strausfeld. Pp. xii+214. (71 plates.) (Springer: Berlin and New York, 1976.) \$118.10.

THE combination of Nick Strausfeld's scientific knowledge and literary and artistic abilities with a large format and high production standards has resulted in an admirable monograph about the neuroanatomy of the fly's brain, the text of which merits much discussion and suggests many experiments. There is also an abundance of photomicrographs and drawings, of high quality, illustrating those exquisite ramifications of nerve cells which are so beloved by anatomists, but which can cause the physiologist to despair sometimes of ever fully comprehending the workings of the nervous system.

Divided into seven chapters and two appendices, the book begins with a historical survey and proceeds to a description of the major cell types found in the fly's brain together with a classification of neurones based on the shapes of the processes of the nerve cells. The third chapter describes the primary compartments of the brain and the fourth, the coordinate system which gives the *Atlas* a stereotaxic frame of reference. The *Atlas* itself, composed of serial sections of the brain stained by reduced silver methods, forms chapter 6 and this is preceded by a chapter which considers some quantitative aspects of brain structure. The final chapter shows the forms and dispositions of various neurones as revealed by variations of the Golgi impregnation technique. One appendix gives a multi-author and multi-lingual dictionary of the terminology of the fly's brain, whereas the other provides valuable information about the various histological techniques which can be applied to the insect nervous system.

The vast amount of anatomical information presented forms an excellent substratum on which to build future experiments and into which previous physiological and behavioural studies can be integrated. The neuroanatomy and possible function are related in a heuristically worthwhile manner. The book is marred only by the high incidence of uncorrected typographical errors.

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## Crystallography

*Space Structures: Their Harmony and Counterpoint.* By Arthur L. Loeb. Pp. xviii+169. (Addison-Wesley: Reading, Massachusetts and London, February 1976.) Cloth \$19.50; paper \$9.50.

THE structure of ordinary Euclidean space is not as simple as it seems. Just as the complexity of chemistry arises from the Pauli exclusion principle, forbidding two electrons to have the same set of quantum numbers, so much of the complex structure of space arises from what we might call the Democritean exclusion principle—no two atoms may have the same set of  $(x, y, z, t)$  space-time quantum numbers (otherwise knots would fall apart).

Arthur Loeb has produced a fascinating book, at about A-level standard, of interest to crystallographers and applied artists working in space. Indeed it is dedicated to all

'philomorphs'—a useful coinage for a spectrum recognisable as including the 'dome-folk' as well as the graph theorists, the cubists and the biomorphologists.

Much of the material is an exposition of the consequences of the Euler-Schläfli relationship ( $\text{faces} + \text{vertices} = \text{edges} + 2$ ), which is at the heart of the phase rule of chemistry and expresses surprisingly powerful constraints—seen at their best in Rhines' and Craig's analysis of grain growth in aluminium (*Metall. Trans.*, **5**, 413–425, 1974). Truncation, stellation and the division of space into Dirichlet domains are also discussed. It is all fairly familiar material, but well-presented without the obscurities with which graph theoreticians so often cloak their theorems, and is thus of wide popular interest.

**A. L. Mackay**

*Dr Mackay is a reader in the Department of Crystallography at Birkbeck College, University of London, UK.*



## Poetic self-portrait

*Moulds of Understanding: a Pattern of Natural Philosophy.* By Joseph Needham. Pp. 320. (Allen and Unwin: London, April 1976.) £7.75.

JOSEPH NEEDHAM is so widely known personally that it must be almost impossible to find a detached reviewer for any of his works. I certainly cannot claim to be detached. This collection of essays confirms one's respect even though it may not convert one to his political convictions. It waves a wide arm over the extensive view he has of ethical imperatives, human love and the sense of what is holy. For this is what these essays are about, even though their titles and their content are scientific in origin and substance.

To call the essays *A Pattern of Natural Philosophy* is to stretch the phrase far beyond its traditional use. They have been selected by Gary Werskey, obviously with a bias towards one aspect of Needham's thought—the political—so that they coalesce into a self-portrait, an animated image of a man who has never forgotten his personal beginnings, but never lowers his sights from the destiny of human kind. Cambridge

has been his immediate world, described in an essay of 1932 in poetic language which acknowledges his debt to those contemporaries who chose poetry itself as their way of life.

To those who know only his Chinese work or the earlier embryology, it will come as a surprise to find orthodox Christian religion playing a large part in the formation of his outlook both then and now. One might expect, of course, to find early evidence of his well-known socialist convictions, which he has exhibited ever since with both style and humility which others might do well to emulate, whatever their political convictions may be. But it is fascinating to find judgements of great weight being derived from an appreciation of the numinous elements in the traditional church liturgy (the exponents of so-called popularised versions of the long familiar liturgy in both Anglican and Roman churches might learn something to their advantage from what Needham has to say on this subject).

The other essays are grouped under the headings 'Understanding' (all three concerned in some way or other with science and religion), 'Process' (three studies of evolution considered in relation to the organisational interpretation of the Second Law of

Thermodynamics), 'Process and Understanding' (three presentations of a view of man, and particularly scientific man, in a future society formed as much by religious enlightenment as by universal socialism). The final essay is a brief attempt to relate the current Chinese experience to what Needham believes are the World's needs.

One could wish to have more of his rebuttal of the fashionable anti-science of today. For, although a socialism we cannot all accept and a religion for which each of us must be free to seek an alternative, give shape to Needham's philosophy, the solid substance of it is universal science, a progressive science whose development in East and West has played a part in the evolution of the human species just as much as any more obviously biological factor.

Apart from anything else it is a most entertaining book to read, because it is full of good humour and courtesy. He treats his reader by the light of one of his favourite Confucian precepts: "Behave to every man as one receiving a great guest". **Frank Greenaway**

*Frank Greenaway is Keeper of the Department of Chemistry at the Science Museum, London, UK.*

## Delight in animal form

*Starfish, Jellyfish, and the Order of Life: Issues in Nineteenth-Century Sciences.* (Yale Studies in the History of Science and Medicine, Vol. 10.) By Mary P. Winsor. Pp. x+228. (Yale University: New Haven and London, January 1976.) £10.50.

MARY PICKARD WINSOR'S *Starfish, Jellyfish and the Order of Life* is the tenth in a series of works developed from PhD theses. Her original study was of "Issues in the Classification of Radiates"—a history of the decline and fragmentation of a miscellany of lower Invertebrates so collected together by Cuvier as one of the four major divisions of the Animal Kingdom. Professor Winsor sees the need "to follow scientists into the farthest corners of their research, lest the historian should mirror uncritically their own self-portrait" and to "go beyond . . . textbooks, prefaces and essays intended for the general public". This book is successfully illustrated by half-tone offset lithos of the (mostly) lithographic plates. There is a lively interest in, and competent assessment of MacLeay's Quinorianism, the early study of Alternation of Generations by the

student priest Michael Sars of Norway and the Dane Johan Japetus Smith Steenstrup. The young, somewhat erratic, but already the probing doubter T. H. Huxley's relationship with W. S. MacLeay is splendidly documented, and there are a whole sequence of discoveries made in the Agassiz Library at Harvard which houses Louis' and his son Alexander's collection of annotated off-prints. That radial starfish had bilateral larvae, as Johannes Müller observed on Heligoland, was a great trial to Louis. Moreover many illustrations of what varieties of meaning lay behind the description "Natural Classification" are presented with sufficient documentation. A rich and full book for historians of science, of marine biology and those zoologists who may still delight in animal form. Darwin was much more closely involved in this story than Dr Winsor realises. Her attempts in her glossary to explain the Animals to the historians range at times from the naive to the grotesque. The bibliographies are remarkably good. The writing has wit and elegance.

**Sydney Smith**

*Sydney Smith is a lecturer in the Department of Zoology at the University of Cambridge, UK.*

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*Spectrophotometric Determination of Elements.* By Zygmunt Marczenko. Pp. xi+643. (Ellis Horwood: Chichester, February 1976.) £19.50; \$42.90.

THIS volume is one of a series on analytical chemistry being produced by Ellis Horwood Ltd. It is based on original Polish (1968) and Russian (1971) editions, but it has been extensively revised and up-dated for the English version, and now includes nearly 7,000 references, up to the end of 1973.

Part I (100pp) deals with the general principles of spectrophotometric analysis, a survey of available spectrophotometric reagents, and a discussion of preconcentration and separation procedures for the elements. Part II (520pp) is a thorough and systematic practical guide to the separation and determination of the individual elements.

The theoretical background to analytical spectrophotometry is briefly but adequately described in Section I, but this is essentially a practical handbook. Descriptions of the determinations of individual elements are presented in a standardised format, and these descriptions are written in a clear and easy-to-follow style. Several possible analytical procedures are outlined in all cases, and the listing of useful colorimetric reagents is extremely thorough.

A useful feature of Marczenko's book for readers in Western Europe and the US is that it covers the Russian and East European literature comprehensively, and includes references to some journals not readily accessible.

The book is well produced, seems to be reasonably free from typographical errors, and although not cheap it should be a useful acquisition for analytical laboratories, or individual research workers needing to use spectrophotometry to analyse for a wide range of elements.

**G. Davidson**

*Migration and Homing in Animals.* (Zoophysiology and Ecology, Vol. 6.) By Klaus Schmidt-Koenig. Pp. xii+99. (Springer: Berlin and New York, 1975.) DM46; \$18.90.

THIS book provides a short, useful summary of the literature, but its excessive price (the text is only 77 small pages) will prohibit its intended audience of laymen and students from buying it. In any case the style of the book makes it more suitable for a reader who is already familiar with some of the original work than for

the whole clear, if uninspired; however, the text is often too terse and dogmatic for the uninitiated. For example, on p48, orientation experiments with turtles and lizards are dismissed as "methodologically . . . not convincing" with no further explanation. Similarly on p65, it is stated as fact that clock-shifting experiments with pigeons refute the sun-arc hypothesis, without explaining why. I would have thought that an introductory book should not only present results but also explain, where possible, the reasoning behind their interpretation.

The organisation of the book is taxonomic: the author works through various groups of arthropods and vertebrates, in each case summarising the field evidence on migration and homing before outlining the experimental work aimed at elucidating

## Books brief

a complete novice. The writing is on mechanisms of orientation or navigation. The literature survey seems to be accurate and fairly up to date (especially the section on birds—the author's own interest), and the text is well illustrated. Inexplicably, the book ends with an appendix on statistical methods for the analysis of orientation data, which is rather esoteric for an introductory book.

**John R. Krebs**

*The Papers of Joseph Henry.* Vol. 2: November 1832–December 1835—The Princeton Years. Edited by Nathan Reingold. Pp. xxxix+524. (Smithsonian Institution: Washington, DC, December 1975.) \$30.

THIS second volume maintains the high standard and interest of the first (for review, see *Nature*, 242, 481–482, 1973). It consists of letters from and to—and occasionally about—Joseph Henry during his early years as a professor at Princeton, interspersed with notebook entries describing his work on electromagnetic induction and other topics. At first there is little about his research; but once really settled in Princeton, and having established contacts with men of science in Philadelphia, Henry got down to work once more. We see

some of the frustrations of working in what was then a provincial situation, far from the great centres of science in Europe; for although Henry anticipated Faraday in some discoveries his work did not quickly become known outside the USA, and it is only as this volume ends that Henry can be seen making contact with physicists in Europe. These volumes, with their very full and helpful footnotes and indices, not only give us an excellent picture of Henry's social and intellectual life, but are particularly valuable also for casting much light on the gradual formation of a scientific community in the US. Historians will turn to these papers to learn not only about Henry, but about men of science and learning, scientific institutions, and technology in his day as well.

**David M. Knight**

*The Food in Your Future: Steps to Abundance.* By Keith C. Barrons. Pp. 180. (Van Nostrand Reinhold: New York and London, November 1975.) £4.

I TOOK an immediate dislike to Dr Barrons' book, and with each chapter my dislike intensified. It is surely unreasonable of him to collect together as enemies of good nutrition everyone from conservationists to the Pope. He is also out of date. One might expect 'The Great Protein War' to be about the reappraisal by nutritionists of the status of protein in the dietary pantheon. To Dr Barrons it is the basis for future agricultural strategy, although he does not seem to be aware it was an obsolescent one a decade ago.

The final chapter, entitled 'You—A Part of the Solution?', crystallised his view. Future world food needs are to be met by technology, and somebody called Johnny Q. Citizen eating less, wasting less and lobbying congress. Decency, democracy and technology, these three—a strange recipe in an era when the Green Revolution has wilted and political cynicism become a way of life. But overall, although Dr Barrons' book is a tedious, stylistic nightmare, his optimism about both the technological fix and the goodness of man is attractive. His claim that agricultural technology has delivered the goods is true. His belief that it would continue to do so is impressive.

I don't like the book, but I admire it for the optimism that pervades it. I shall therefore read it again, and I recommend my colleagues to do the same.

**John Rivers**

# obituary

**Leonard Ary Woodward** died on June 5 at the age of 73. After starting at Oxford reading mathematics, he switched to chemistry for his final degree, and then went to Leipzig for his Ph.D. (1931), where he developed his lifelong interest in spectroscopy.

He came back to the UK, and, after working for a time at the DSIR, returned to Oxford in 1939, where he was to remain for the rest of his career. It was here that, during the war, he worked on the separation of uranium isotopes by diffusion, research which was to have such influential results. After the war, though, he resumed his work on Raman spectroscopy, a field to which he made major theoretical and experimental contributions. He was made the Aldrichian Praelector in Chemistry in 1966 and served for many years as Dean and then Vice-Principal of Jesus College.

**Victor Ivor Little**, reader in experimental physics at Royal Holloway College, University of London, died on

May 30 at the age of 56.

On graduating, in 1941 from King's College, London, he was swept up in the war effort, working on radar and the countermeasures to the V1 and V2. This experience confirmed his feelings of the practical importance of physics, an enthusiastic belief he later expressed in some measure by his work, as Chairman of the Examination Board, in restructuring the London 'A' level exams. He was also a brilliant lecturer.

His major scientific work was done in the field of non-linear optics; he early realised the exciting research possibilities of the laser. He was the first to show that standing laser radiation in fluids could create a three-dimensional grating—like a Bragg crystal grating, but on a much larger scale—and, more recently, he contributed to the development of very intense liquid laser sources.

**William Bernard Crow, FLS, FZS, FRSM**, died on June 28 at the age of 80. In a varied career as biologist

and anthropologist, he studied at the Universities of London and Wales, worked for the Ministry of Munitions in the First World War, returned to Cardiff as a lecturer, became head of the department of Biology at Huddersfield Technical College, senior lecturer at the South West Essex Technical College, a lecturer in adult education (Northern Command) during the Second World War, went to the Leicester College of Technology and finally became Head of the Department of Biology at the Davies, Laing and Dick Tutorial College.

His major scientific interest was in morphology. He published much research in this field as well as a textbook, *Contributions to the Principles of Morphology* (1929). His broad outside interests, however, are reflected in the titles of some of his other publications: *Voice and the Vocal Apparatus*; *The Science of Dreams*, *A Synopsis of Biology*, *A History of Magic, Witchcraft and Occultism* and *Occult Properties of Herbs*.

## announcements

### Appointments

**Professor J. E. Enderby** to the Chair of Physics at the University of Bristol. **Dr H. K. Moffatt** to the Chair of Applied Mathematics at the University of Bristol.

**Professor D. L. Gardner** to the Chair of Histopathology at the University of Manchester.

**Dr T. B. Grimley** and **Dr A. Ledwith** as Professors of Chemistry at the University of Liverpool.

### Awards

The EPS Hewlett-Packard Europhysics Prize has been awarded to **Professor Wolfgang Helfrich** of the Freie Universität, Berlin for work on the physics of Liquid Crystals.

The President of the USA has awarded the National Medal of Science to: **John W. Backus** of San Francisco; **Manson Benedict** of Weston, Massachusetts; **Hans A. Bethe** of Ithaca, New York; **Shiing-Shen Chern** of El Cerrito, California; **George B. Dantzig**

of Stanford, California; **Hallowell Davis** of St Louis, Missouri; **Paul Gyorgy** (posthumous award); **Sterling Brown Hendricks** of Silver Spring, Maryland; **Joseph O. Hirschfelder** of Madison, Wisconsin; **William H. Pickering** of Pasadena, California; **Lewis H. Saret** of Skillman, New Jersey; **Frederick E. Terman**, of Stanford, California, **Orville Alvin Vogel** of Pullman, Washington; **E. Bright Wilson, Jr** of Concord, Massachusetts and **Chien-Shiung Wu** of New York.

The Simon Memorial Prize of the Institute of Physics has been awarded to **Drs D. M. Lee, D. Osheroff** and **R. C. Richardson** for their work on the superfluid phase of  $^3\text{He}$ .

### Meetings

August 21–27, **Birth Defects**, Montreal (Deadline for abstracts: March 1, 1977) (International Birth Defects Congress Ltd, 1275 Mamoroneck Avenue, White Plains, New York 10605).

September 7–10, **Meeting of the Euro-**

**pean Geophysical Society**, Amsterdam (3rd EGS Meeting (Registration), c/o Vrije Universiteit, de Boelelaan 1105, Amsterdam).

September 13–15, **Solar Energy in Agriculture**, Reading (The Secretary, UK-ISES, The Royal Institution, 21 Albemarle Street, London W1X 4BS).

September 26–28, **Planning for Water and Waste in Hot Countries**, Loughborough (John Pickford, University of Technology, Loughborough, Leics. LE11 3TU, UK).

September 29–October 1, **Comparative Planetology**, College Park (Dr Cyril Ponnamperna, Laboratory of Chemical Evolution, Department of Chemistry, University of Maryland, College Park, Maryland 20742).

September 30–October 1, **Pacific NW Regional Meeting of the AGU**, Victoria, British Columbia (Deadline for abstracts: August 27) (John T. Weaver, Dept of Physics, University of Victoria, Victoria, B.C., Canada V8W 2Y2).

October 18–20, **Protein Synthesis**,



Bethesda, Maryland (Dr William Merri-  
rick, Symposium Director, Building 10,  
Room 7D-18, NIH, Bethesda, Mary-  
land 20014).

October 28, **Sources for Emission Spec-**  
**troscopy**, Middlesbrough (The Analy-  
tical Division, Chemical Society,  
Burlington House, London W1V 0BN).

November 17-18, **Ozone/Chlorine Di-**  
**oxide Oxidation Products of Organic**  
**Materials**, Cincinnati (Dr Rip G. Rice,  
International Ozone Institute, Syracuse  
University, New York 13210).

November 24, **Synthesis of Cyclic Com-**  
**pounds**, London (The Assistant Secre-  
tary, Society of Chemical Industry, 14  
Belgrave Square, London SW1 8PS).

November 24, **Applications of Fluori-**  
**metry to Chromatography**, Guildford  
(Mr D. Irish, UV Group, c/o Pye  
Unicam Ltd, York Street, Cambridge).

December 10, **Observation of Surfaces**  
**and Surface Reactions by TEM**, Lon-  
don (Deadline for abstracts to Dr  
W. M. Stobbs, Cavendish Laboratory,  
Madingley Road, Cambridge CB3  
0ME: November 1) (The Meetings  
Officer, The Institute of Physics, 47  
Belgrave Square, London SW1 8QX).  
December 13-15, **Optical Properties**  
**and Effects of Atmospheric Aerosols**,  
Williamsburg, Virginia (Deadline for  
papers: September 10) (Franklin S.  
Harris, Jr, MS475, NASA Langley  
Research Center, Hampton, Virginia  
23665).

January 10-14, 1977, **Molecular Clon-**  
**ing of Recombinant DNA and Genetic**  
**Manipulation as it affects the Cancer**  
**Problem**, Miami (Miami Winter Sym-  
posia, PO Box 520906, Miami, Florida  
33152).

March 29-31, 1977, **Luminescence Pro-**  
**cesses in Phosphors for Cathode-Ray**  
**Tubes and Lamps**, Weybridge, Surrey  
(Dr P. Greenough, General Electric Co  
Ltd, Hirst Research Centre, East Lane,  
Wembley, Middlesex HA9 7PP, UK).  
April 25-29, 1977, **Mitosis: Facts and**  
**Questions** (Deadline for applications:  
September 30) (Organising Committee,  
Kennwort: Mitosis, Institut für Zell-  
forschung, Deutsches Krebsforschungs-  
zentrum, PO Box 101949, D-6900  
Heidelberg, FRG).

June 1-3, 1977, **Pathobiology of En-**  
**vironmental Pollutants**, University of  
Connecticut (Deadline for abstracts:  
January 1, 1977) (George Migaki,  
D.V.M., Registry of Comparative Path-  
ology, Armed Forces Institute of Path-  
ology, Washington, D.C. 20306).

July 6-8, 1977, **Hot Electrons in Semi-**  
**conductors**, Denton, Texas (Deadline  
for abstracts: March 1, 1977) (Dr D. G.  
Seiler, Physics Dept, North Texas  
State University, Denton, Texas 76203).  
August 9-19, 1977, **IASPEI/IAVCEI**

## Person to Person

The Superconducting Magnet Re-  
search Group at the SRC Ruther-  
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superconductor testing facilities  
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Assembly, Durham (Dr R. E. Long,  
IASPEI/IAVCEI Assembly Office,  
University of Durham, Dept of Geo-  
logical Sciences, Science Laboratories,  
South Road, Durham DH1 3LE, UK).

September 4-9, 1977, **4th International**  
**Congress of Photosynthesis**, Reading  
(Dr J. Coombs, c/o Tate and Lyle Ltd,  
Group Research and Development, PO  
Box 68, Reading RG6 2BX, Berks,  
UK).

September 6-9, 1977, **Plant Cell and**  
**Tissue Culture: Principles and Appli-**  
**cations**, Columbus, Ohio (Fourth  
Annual Colloquium, College of Bio-  
logical Sciences, 484 W. 12th Avenue,  
The Ohio State University, Columbus,  
Ohio 43210).

September 19-22, 1977, **Electron Dif-**  
**fraction**, London (The Meetings  
Officer, The Institute of Physics, 47  
Belgrave Square, London SW1 8QX).  
September 19-24, 1977, **Availability**  
**and Rational Uses of Energy Resources**,  
Istanbul (The Secretary General of the  
World Energy Conference, 5 Bury  
Street, St James's, London SW1Y  
6AB, UK).

October 31-November 3, 1977, **Advan-**  
**cing Energy Technology**, Eastbourne,

Sussex (Deadline for abstracts: Janu-  
ary 3, 1977) (Prof A. Williams, The  
University of Leeds, Leeds LS2 9JT,  
UK).

## Reports and Publications

### Gt Britain

Bulletin of the British Museum (Natural History).  
Entomology. Vol. 33, No. 1 + The Classification of the  
Anomaloniinae (Hymenoptera: Ichneumonidae). By  
Ian David Gauld. Pp. 1-135. (London: British Museum  
(Natural History), 1976.) £10.70. [233]

Memoirs of the Royal Astronomical Society. Vol. 81,  
Part 2: Polarimetric Observations of the Zodiacal  
Light in Hawaii from 1969 to 1974. By L. W. Bander-  
mann and R. D. Wolstencroft. The Nebular Complexes  
of the Large and Small Magellanic Clouds. By R. D.  
Davies, K. H. Elliott and J. Meaburn. Pp. 37-128 +  
21 plates. (Oxford and London: Blackwell Scientific  
Publications, 1976. Published for the Royal Astronomi-  
cal Society.) [233]

Superstar Technologies. (The problem of monitoring  
technologies in those instances where technical com-  
petence is monopolised by a small number of institu-  
tions committed to the same interest.) The Report of  
a Working Party. Convenor: Geoffrey Chin. Council  
Representative: Professor John Ziman, FRS. Pp. 66.  
(Chichester: Barry Rose (Publishers), Ltd., in associa-  
tion with The Council for Science and Society,  
London.) £2. [243]

Imperial Cancer Research Fund. Annual Report  
and Accounts, 1975. Pp. 41. Scientific Report. Pp. 162.  
(London: Imperial Cancer Research Fund, 1976.) [243]

University of Oxford. Annual Reports, 1973/1974.  
Pp. 12. (Oxford: The University, 1976.) 75p. [263]

The Small Towns of Hampshire: The Archaeological  
and Historical Implications of Development. By  
Michael Hughes. Pp. iv + 148. (Southampton:  
Hampshire Archaeological Committee, Department  
of Archaeology, The University, 1976.) £1.50. [263]

Radio, Television and the Arts. By Howard Newby.  
(BBC Lunch-Time Lectures, Tenth Series, No. 4.)  
Pp. 16. (London: BBC, 1976.) [293]

Bulletin of the British Museum (Natural History).  
Entomology. Vol. 33, No. 2: A Review of the Genus  
*Anotylus* G. Thomson (Coleoptera: Staphylinidae).  
By P. M. Hammond. Pp. 137-187 + 3 plates. (London:  
British Museum (Natural History), 1976.) £4.35. [293]

Natural Environment Research Council. Institute  
of Geological Sciences. Geomagnetic Bulletin No. 5:  
Magnetic Results 1971-Eskdalemuir, Hartland and  
Lerwick Observatories. Pp. vi + 97. £4.50. Geomag-  
netic Bulletin No. 6: Annual Mean Values  
of Geomagnetic Elements since 1941. Pp. 170. £7.  
(London: HMSO, 1975 and 1976.) [293]

Proceedings of the Royal Irish Academy. Vol. 76,  
Section A. No. 1: On the Coefficients in the Recursion  
Formulae of Orthogonal Polynomials. By G. Freud.  
Pp. 1-6. 45p. No. 2: A Note on Complementarity in  
Lattices of Convergence Functions. By C. A. Boyd.  
Pp. 7-10. 25p. No. 3: A Note on the Reflection  
Principle for Harmonic Functions. By D. H. Armitage.  
Pp. 11-14. 28p. No. 4: The Ideal Centre of a Banach  
Lattice. By A. W. Wickstead. Pp. 15-24. 48p. No. 5:  
Haar Measures for Groups. By A. K. Seda. Pp. 25-36.  
48p. No. 6: On the Construction of the Kerr  
Congruence. By P. A. Hogan. Pp. 37-42. 45p. No. 7:  
Relaxation Effects in Rotational Brownian Motion.  
By J. T. Lewis, J. McConnell and B. K. P. Scaife. Pp.  
43-70. 92p. No. 8: Modified Kohn Variational  
Principle, The Virial Theorem and the Second Virial  
Coefficient. By W. D. Burns and B. L. Moiseiwitsch.  
Pp. 71-78. 50p. Vol. 76, Section B. No. 4: Stratigraphy  
Sedimentology and Structure of the Bray Group in  
County Wicklow and South County Dublin. By P. M.  
Bruck and T. J. Reeves. Pp. 53-78 + plates 1-3. 87p.  
No. 5: Formation of Oxo-Compounds from Chloro-  
benzodiazines in Dimethylsulphoxide. By D. Twomey.  
Pp. 79-86. 25p. No. 6: A Preliminary List of the Fungus-  
Gnats (Diptera: Mycetophilidae) of Ireland. By F. J.  
Chandler. Pp. 87-110. 66p. No. 7: The Submarine  
Extensions of the Thorr and Fanad Plutons, County  
Donegal. By D. Evans and R. J. Whittington. Pp.  
111-120. 40p. (Dublin: Royal Irish Academy, 1976.)  
[303]

### Other countries

US Department of the Interior: Geological Survey.  
Bulletin 1395-E: Changes in Nomenclature of Upper  
Precambrian to Lower Paleozoic(?) Formations in the  
Natick Quadrangle, Eastern Massachusetts, and  
Their Tentative Correlations with Rocks in Rhode  
Island and Connecticut. By A. E. Nelson. Pp. iii + 15.  
(Washington, DCL US Government Printing Office,  
1974.) 35 cents. [303]

Toxicology Research Projects Directory, Vol. 1,  
No. 1, January 1976. (An Indexed Directory of Current  
Research Project Abstracts in Toxicology and Related  
Fields.) Pp. 701. (Bethesda, Md.: Toxicology Informa-  
tion Subcommittee of the DHEW Committee to Coordi-  
nate Toxicology and Related Programs, 1976. Orders to  
Toxicology Document and Data Depository, National  
Technical Information Service, Springfield, Virginia,  
22161. Specify Publication No. NTISUB/B/021-76/  
001.) Single issue \$20. Subscription (4 issues) \$65. [313]

Smithsonian Contributions to Zoology, No. 213:  
Communication Mechanisms and Social Integration in  
the Black Spider Monkey, *Atelés fusciceps robustus*,  
and Related Species. By John F. Eisenberg. Pp. vi +  
108. (Washington, DC: Smithsonian Institution Press,  
1976. For sale by US Government Printing Office.) [313]